BLACKEYE COWPEA MOSAIC VIRUS: PURIFICATION, PARTIAL CHARACTERIZATION, SEROLOGY, AND IMMUNOCHEMICAL AND CYTOLOGICAL TECHNIQUES FOR DETECTION OF VIRUS-INFECTED LEGUME SEEDS

By

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To my wife, Diana, and my son, Roberto, who with understanding, friendship, and love helped to transform a goal into a reality.

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VIRUS ABBREVIATIONS

Virus Names	Abbreviation
Bean common mosaic virus	BCMV
Bean common mosaic virus-siratro isolate	BCMV-S
Bean pod mottle virus	BPMV
Bean yellow mosaic virus	BYMV
Bidens mottle virus	BiMV
Blackeye cowpea mosaic virus	BlCMV
Clover yellow vein virus	CYVV
Commelina mosaic virus	CoMV
Cowpea aphid-borne mosaic virus	CAMV
Cowpea chlorotic mottle virus	CCMV
Cowpea mild mottle virus	CMMV
Cowpea mosaic virus	CPMV
Cowpea ringspot virus	CpRV
Cowpea yellow mosaic virus	CYMV
Cucumber mosaic virus	CMV
Dasheen mosaic virus	DMV
Iris mosaic virus	IMV
Lettuce mosaic virus	LMV
Pea seed-borne mosaic virus	PSMV
Pepper mottle virus	PeMV
Pepper vein mottle virus	PVMV
Pokeweed mosaic virus	PWMV
Potato virus X	PVX
Potato virus Y	PVY
Southern bean mosaic virus	SBMV
Soybean mosaic virus	SoyMV
Sugarcane mosaic virus	SMV
Tobacco etch virus	TEV
Tobacco mosaic virus	TMV
Tobacco ringspot virus	TRSV
Turnip yellow mosaic virus	TuMV
Watermelon mosaic virus-1	WMV-1
Watermelon mosaic virus-2	WMV-2

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Blackeye cowpea mosaic virus (BlCMV) was increased in cowpea, Vigna unguiculata (L.) Walp., 'Knuckle Purple Hull', and infected leaves were used for virus and cytoplasmic inclusion purification. Either n-butanol or a combination of chloroform and carbon tetrachloride was used in the clarification process. Polyacrylamide gel electrophoresis of sodium dodecyl sulfate (SDS) dissociated inclusions and virus revealed that the inclusions were made of a single protein estimated to have a molecular weight (MW) around 70,000 daltons whereas freshly purified BlCMV consisted of a main protein component with a MW of 34,000 daltons and two smaller proteins with MWs of 29,000 and 27,000 daltons. Purified BlCMV had a 260/280 nm absorption ratio of 1.2 and a modal length of 753 nm. Freshly purified BlCMV preparations showed a single sedimenting peak with s_{20}=157-159 S. The purified BlCMV cytoplasmic inclusions had absorption spectra characteristic for proteins. Electron microscopy of purified inclusions revealed the presence of tubes showing striations with periodicities of approximately 5 nm.

Antisera reactive in SDS-immunodiffusion were obtained against untreated virions, pyrrolidine degraded coat protein, and untreated BlCMV cytoplasmic inclusions. Reciprocal double immunodiffusion tests with SDS-treated antigens showed that BlCMV is serologically unrelated to seven potyviruses and serologically related to, but distinct from: bean common mosaic virus (BCMV), bean yellow mosaic virus (BYMV), cowpea aphid-borne mosaic virus (CAMV), dasheen mosaic virus (DMV), lettuce mosaic virus (LMV), potato virus Y (PVY), soybean mosaic virus (SoyMV), tobacco etch virus (TEV), and watermelon mosaic virus-2 (WMV-2). The intragel cross-absorption technique was also used to demonstrate distinction between closely related potyviruses. Agar medium impregnated with a mixture of antisera was used for serodiagnosis of BlCMV and cowpea mosaic virus in cowpea.

Light and electron microscopy of cytoplasmic inclusions induced by BlCMV, siratro (Macroptilium atropurpureum (D.C.) Urb.) strain of BCMV (BCMV-S) and CAMV revealed that they are similar to those induced by the potyviruses from Edwardson's subdivision-I. The different reactions induced by BlCMV, BCMV-S, and CAMV in some cowpea varieties indicated that they can also be used as differential hosts for these three potyviruses. Sources of resistance for BlCMV were found among the cowpea varieties tested. Based on its physical, biological, cytological, and immunochemical properties, BlCMV can be differentiated from any other virus that infects cowpea.

Cytoplasmic inclusions induced by BlCMV in cowpea and by SoyMV in soybean were detected by serology, light microscopy, and electron microscopy in hypocotyls of 4-5-day-old seedlings grown from virus-infected seeds.

Immunodiffusion tests and serologically specific electron microscopy were used to detect BlCMV in hypocotyls of 4-5-day-old cowpea seedlings grown from BlCMV-infected seeds. Discs of individual hypocotyls were embedded into the agar medium 4-5 mm away from the antiserum wells. Virus-specific precipitin lines formed between virus-infected hypocotyl discs and antiserum wells, whereas no reactions were observed with healthy hypocotyls. Precipitin lines were also observed with extracts of mixtures from infected (1 g) and healthy (up to 29 g) tissues. These immunochemical techniques were also used for detecting BCMV in hypocotyls of infected 4-5-day-old Phaseolus vulgaris L. seedlings and for detecting SoyMV in infected Glycine max (L.) Merr. seedlings. Single radial immunodiffusion tests with extracts or discs of cowpea hypocotyls were also useful for detecting BlCMV in germinated seeds. The reliability and simplicity of the immunodiffusion tests make them suitable for use in routine seed health testing program in any laboratory.

CHAPTER I

PURIFICATION, PARTIAL CHARACTERIZATION, AND SEROLOGY OF BLACKEYE COWPEA MOSAIC VIRUS

Introduction

Cowpea, Vigna unguiculata (L.) Walp. (=Vigna sinensis (L.) Endl.), is grown as a crop in high-temperature areas of tropical and subtropical countries. Cowpea seeds constitute a source of good quality protein and dried seeds are an important part of the diet of many people in the tropical and subtropical world, particularly in Africa and the rural zone of northeastern Brazil. The fresh seeds and immature pods are also eaten and they can be frozen or canned as is sometimes done in the United States. Cowpeas are also grown as fodder plants for hay, silage or pasture and used as a green manure and cover crop. When grown under optimum conditions, cowpea can produce seed yields as high as 2,600 Kg/ha. However, several factors limit cowpea yields in most fields. Virus diseases are considered as a major limiting factor to the production of cowpeas in several countries (Dale, 1949; Wells and Deba, 1961; Toler et al., 1963; Brantley et al., 1965; Kuhn et al., 1966; Harrison and Gudauskas, 1968a; Harrison and Gudauskas, 1968b; Gay and Winstead, 1970; Zettler and Evans, 1972; Bock, 1973; Phatak, 1974; Haque and Persad, 1975; Kaiser and Mossahebi, 1975; and Lima and Nelson, 1977). Several viruses infect cowpea, and many of them can be transmitted through seeds from infected cowpea plants. The most important cowpea seed-borne virus in the southeastern United States is

an aphid-transmitted, filamentous virus approximately 750 nm long (Harrison and Gudauskas, 1968a; 1968b; Gay and Winstead, 1970; Zettler and Evans, 1972; and Uyemoto et al., 1973). This virus was first isolated in Florida by Anderson (1955a), who designated it "blackeye cowpea mosaic virus" (BlCMV) (Anderson, 1955b), a name that has been retained by Zettler and Evans (1972) and Edwardson et al. (1972).

Because no antiserum specific for BlCMV was available, and because only sparse information about the virus properties could be found in the literature, the first part of this research was undertaken to purify and characterize BlCMV in vitro and in vivo. Antisera prepared for BlCMV and its cytoplasmic inclusions were used for serological characterization of the virus. Some methods for virus and inclusion purification, as well as certain physical, biological, immunochemical and cytological properties of BlCMV were described in the present investigation. An abstract of part of this research was already published (Lima et al., 1976).

Literature Review

Several viruses infect cowpea, V. unguiculata, causing different types of mosaic. The first report about mosaic of cowpea was published in 1921 by Elliot, who reported a high incidence of cowpea virus disease in Arkansas (Elliot, 1921). Smith (1924) demonstrated experimentally that the cowpea virus was transmitted either by rubbing the leaves of diseased and healthy plants together or by the bean leaf beetle, Ceratoma trifurcata Forst. Subsequently, Gardner (1927) working with a cowpea virus, observed that it was transmitted through seeds of certain cowpea varieties.

A widespread mosaic disease was reported on different cowpea varieties in Trinidad (Dale, 1949). Dale (1949) observed that the virus responsible for the disease was not transmitted by Aphis medicaginis Koch, but that the leaf beetle, Ceratoma ruficornis (Oliv.) was a good vector and was probably responsible for transmitting the virus in the field. On the basis of his studies, he concluded that the virus was unrelated to those described by McLean (1941), Snyder (1942), and Yu (1946), but was more likely the virus studied by Smith (1924). Dale (1953) subsequently confirmed that the cowpea mosaic virus isolated from Trinidad was efficiently transmitted by C. ruficornis, but not by aphids.

Lister and Thresh (1955) isolated a virus from cowpea and identified it as a strain of tobacco mosaic virus (TMV). They observed that a purified preparation of the virus contained rod-shaped particles of varying lengths, indistinguishable from the particles of TMV, and was precipitated specifically with antiserum prepared against TMV. A cowpea strain of TMV was also isolated from a range of leguminous hosts at Ibadan, Nigeria (Chant, 1959). Chant (1959) also found another virus infecting cowpea in Nigeria and as its physical properties differed from other cowpea viruses, he proposed the name cowpea yellow mosaic virus (CYMV). The virus was purified and an antiserum prepared against it. Both TMV and CYMV were transmitted by the beetle Ootheca mutabilis Sahlb. In subsequent work, Chant (1960) studied the influence of TMV and CYMV on growth rate and yield of cowpea, and found that infection of cowpea with the cowpea strain of TMV did not affect yield as much as infection with CYMV. Wells and Deba (1961) tested 116 cowpea varieties and 342 indigenous pure lines against CYMV and observed

that 6 varieties and 16 pure lines were resistant. Robertson (1965) screened 79 cowpea varieties for resistance to CYMV in a screened greenhouse. Those varieties that showed no local or systemic reactions when inoculated with the virus were classified as immune; those that developed necrotic lesions but did not become systemically infected were classified as resistant; and those that showed systemic infection were classified as susceptible. Chant (1962) found that the cowpea virus from Trinidad caused local lesions on Chenopodium amaranticolor Coste and Reyn., Mucuna atterrina Holland, Petunia hybrida Vilm., and P. vulgaris, and that the virus was polyhedral with a mean diameter of approximately 25 nm.

Double-immunodiffusion tests showed that a cowpea virus from Arkansas and the Trinidad cowpea mosaic virus were closely related, but not identical serologically and that both were antigenically related to bean pod mottle virus (BPMV) (Shepherd, 1963). Studying other properties of the virus, Shepherd (1964) confirmed a close similarity of the Arkansas virus with the cowpea mosaic virus from Trinidad (Dale, 1949). Walters and Barnett (1964), working with a cowpea mosaic virus serologically identical to the Arkansas isolate, demonstrated also that it was efficiently transmitted by the bean leaf beetle, C. trifurcata. A detailed study of three cowpea mosaic virus isolates from Surinam (South America), along with the previously reported cowpea viruses from Trinidad (Dale, 1949) and Nigeria (Chant, 1959, 1960, 1962) revealed that they are strains of cowpea mosaic virus (Agrawal, 1964). Detailed descriptions of host range, biophysical, biochemical, and immunochemical properties of cowpea mosaic virus were reported, and the abbreviation CPMV was proposed to eliminate any possible confusion

with CMV (cucumber mosaic virus). Cowpea mosaic virus (CPMV) has been extensively studied in different laboratories and was fully described by van Kammen (1971, 1972). It was selected as the type member of the comovirus group (Fenner, 1976) and reported from several other parts of the world, including Brazil (Carner et al., 1969; and Lima and Nelson, 1977), Nigeria (Williams, 1975), Venezuela (Debrot and Rojas, 1967), and Puerto Rico (Perez and Cortes-Monllor, 1970; and Alconero and Santiago, 1973).

Kuhn (1964b) purified and characterized a new virus isolated from cowpea in Georgia and named it cowpea chlorotic mottle virus (CCMV), which was subsequently described by Bancroft (1971). This virus belongs to the bromovirus group (Fenner, 1976) and is physically similar to brome mosaic virus (Bancroft, 1970) and broad bean mottle virus (Gibbs, 1972), neither of which produces symptoms in cowpea (Bancroft, 1971).

Strains of cucumber mosaic virus (CMV) are also known to infect cowpea. Cucumber mosaic virus strains have been isolated from naturally infected cowpeas showing mosaic symptoms in southeastern United States (Anderson, 1955a; Kuhn, 1964a; and Harrison and Gudauskas, 1968a), Italy (Vovlas and Avgelis, 1972), Morocco (Fischer and Lockhart, 1976b), and South Africa (Klesser, 1960). An aphid-transmitted, spherical virus, approximately 25 nm in diameter, was also reported from India by Chenulu et al. (1968). According to their descriptions, the virus closely resembles a strain of CMV.

Shepherd and Fulton (1962) identified a seed-borne virus of cowpea as a strain of southern bean mosaic virus (SBMV) (Shepherd, 1971). Although a virus isolated from naturally infected cowpea in Arkansas

had properties somewhat similar to the cowpea strain of SBMV, the two viruses were not serologically related (Shepherd, 1963).

A carlavirus isolated from cowpea in Ghana was described and designated as cowpea mild mottle virus (CMMV) by Brunt and Kenten (1973) and Brunt (1974). Cowpea mild mottle virus is seed-borne in cowpeas, is 650 nm in length and is apparently not transmitted by aphids.

A virus with small isometric particles, isolated from Iranian cowpea seeds was considered as new and named cowpea ringspot virus (CpRV) on the basis of symptomatology and particle morphology, which were similar to other ringspot viruses (Phatak, 1974; and Phatak et al., 1976). According to Phatak (1974), the virus was not transmitted by aphids, induced intracellular inclusions in cowpea, had a wide experimental host range and was serologically unrelated to 40 other isometric viruses most of which commonly infect various legumes. Cowpea ringspot virus was also transmitted in 15-20% of the seeds of three cowpea cultivars (Phatak et al., 1976).

McLean (1941) studied some physical and biological properties of a cowpea virus and found that it was transmitted by the following species of aphids: Macrosiphum solani Ashm., Acynthosiphon pisum (Harris), Aphis gossypii Glover, Myzus persicae (Sulz.), but not by the bean leaf hopper (Empoasca fabae Le. B.), the tarnished plant bug (Lygus pratensis L.), the Mexican bean beetle (Epilachra corrupta Mls.), and the striped cucumber beetle (Diabrotica vittata Faba). Snyder (1942) described a mosaic disease of asparagus bean, Vigna sesquipedalis Wight, and also studied some biological and physical properties of the causal agent. His positive results obtained with aphid transmission

indicated that these viruses were not identical to the one described by Smith (1924). A cowpea virus similar to those described by McLean (1941) and Snyder (1942) was reported from China by Yu (1946). The virus which was transmitted by aphids was also seed-borne in cowpea. In addition to cowpea, the virus also infected lima bean and adzuki bean, Phaseolus angularis (Willd.) Wight (=Vigna angularis (Willd.) Ohwi. and Ohshi) (Yu, 1946). Cowpea viruses apparently similar to those were also reported from Ceylon (Abrygunawardena and Perera, 1964), Germany (Brandes, 1964), India (Nariani and Kandaswany, 1961), and New Guinea (van Velsen, 1962).

An aphid-borne virus isolated from cowpea in northern Italy was studied by Lovisolo and Conti (1966), and designated as cowpea aphid-borne mosaic virus (CAMV). The virus was a rod, approximately 750 nm long, and was seed-borne in cowpea, but appeared to be clearly different from BICMV isolated in Florida (Anderson, 1955b). As reported by Lovisolo and Conti (1966), the virus was first recorded and described in Italy by Vidano (1959) and Rui (1960). The virus was transmitted in a non-persistent manner by M. Persicae, Aphis fabae Scop., A. medicaginis Koch, A. gossypii, and Macrosiphum euphorbiae (Thomas) (Vidano and Conti, 1965). A similar virus was later isolated in East Africa and three strains of this virus were differentiated by host range and serology (Bock, 1973). It was also observed that CAMV is distantly serologically related to bean common mosaic virus (BCMV) (Lovisolo and Conti, 1966; and Bock, 1973), but no direct serological relationship was detected with the African type strain of CAMV and potato virus Y (PVY), bean yellow mosaic virus (BYMV), pea seed-borne mosaic virus (PSMV), clover yellow vein virus (CYVV), soybean mosaic virus (SoyMV), sugarcane mosaic virus (SMV),

tobacco severe etch virus (TEV), and iris mosaic virus (IMV) (Bock, 1973; and Bock and Conti, 1974). A seed-transmitted virus tentatively identified as CAMV was considered to be responsible for the most important and widespread disease of cowpeas in Iran (Kaiser et al., 1968). Additional studies about various properties of the Iranian isolate of CAMV indicated its similarity to the Italian and African isolates (Kaiser and Mossahebi, 1975). A CAMV isolate was also reported from Japan infecting adzuki bean, P. angularis, under natural conditions (Tsuchizaki et al., 1970). Fisher and Lockhart (1976a) isolated a rod-shaped virus from severely infected cowpeas in Morocco and identified it as a strain of CAMV on the basis of its particle length, aphid-transmission, host range, serology, and physical properties. The Moroccan isolate differed from those CAMV isolates previously described (Lovisolo and Conti, 1966; Bock, 1973; and Bock and Conti, 1974) by failing to infect Ocimum basilicum L., a diagnostic species for CAMV (Bock and Conti, 1974), and other plants reported to be systemic hosts for CAMV. Padma and Summawar (1973) indicated the value of Chenopodium murale L. as a good indicator host for differentiation, screening and isolation of a rod-shaped cowpea virus and the icosahedral CPMV. Cytoplasmic inclusions were observed in plant cells infected with CAMV (Inouye, 1973; and Nicolaeseu et al., 1976).

A virus isolated from cowpea in India (Khatri and Singh, 1974) was reported to be a strain of CPMV. However, the authors reported aphid transmission of this virus, so its identification as a strain of CPMV is questionable. A filamentous virus approximately 750 nm in length isolated from cowpeas in Ghana did not react with antisera specific for CAMV, peanut mottle virus, BCMV, and BYMV (Brunt, 1974).

An aphid-transmitted virus was responsible for complete loss of cowpea in irrigated areas of northern Nigeria (Raheja and Leleji, 1974). Based on the fact that the virus was neither mechanically transmitted nor seed-borne in cowpea, Raheja and Leleji (1974) concluded that it was either an atypical strain of CAMV or a new virus not previously described.

A virus isolated from Crotalaria spectabilis Roth in a field at Gainesville, Florida, was studied by Anderson (1955b) and designated blackeye cowpea mosaic virus (BICMV). Anderson (1955b, 1955c) reported that BICMV infected plants of cowpea, Crotalaria and Desmodium in the field, but considered Crotalaria and Desmodium as secondary hosts for the virus. In a subsequent study, Anderson (1959) observed that BICMV was transmitted by M. persicae but not by the bean leaf beetle, C. trifurcata. Corbett (1956) found that BICMV was serologically related to BYMV and identified it as a strain of BYMV. Based on Corbett's conclusion, several subsequent reports have referred to BICMV as a cowpea strain of BYMV (Brierly and Smith, 1962; Kuhn, 1964a; Kuhn et al., 1965; and Harrison and Gudauskas, 1968a).

Light and electron microscopic studies of BICMV and BYMV showed marked cytological differences between these two flexuous rod-shaped viruses (Edwardson et al., 1972). According to Edwardson et al. (1972), the cytoplasmic inclusions induced by BICMV were consistently different from those induced by BYMV. In the light microscope, groups of plates were observed in cells of BYMV-infected tissues, whereas groups of tubes were seen in cells of epidermal strips obtained from BICMV-infected tissue. Electron microscopy of ultrathin sections indicated that BICMV-induced cytoplasmic inclusions consisted of pinwheels with scrolls, whereas BYMV-induced cytoplasmic inclusions were made of pinwheels and

laminated aggregates. Light and electron microscopic investigations revealed that BlCMV induces nuclear inclusions in C. spectabilis (Zettler et al., 1967; Edwardson et al., 1972; and Christie and Edwardson, 1977), while no such inclusions were observed in cells of C. spectabilis infected with BYMV. Based on those cytological distinctions, Edwardson et al. (1972) concluded that BlCMV and BYMV are distinct members of the potyvirus group. Subsequently, Zettler and Evans (1972) demonstrated that BlCMV and BYMV had dissimilar host ranges, providing additional evidence that they are distinct viruses.

In host range studies, BlCMV was shown to be very similar to BCMV, but different from watermelon mosaic virus-2 (WMV-2), (Uyemoto et al., 1973). Leaf-dip preparations of BlCMV-infected tissue revealed the presence of flexuous rods, 750 nm long, and double immunodiffusion tests with BCMV and WMV-2 antisera indicated that BlCMV was serologically identical to BCMV and related to, but distinct from WMV-2 (Uyemoto et al., 1973).

Materials and Methods

Source of Virus Isolate

The blackeye cowpea mosaic virus used in this study was isolated from infected seeds of cowpea V. unguiculata 'Knuckle Purple Hull' harvested from a field in Gainesville, Florida. The virus was transmitted by aphids from infected cowpea plants grown from infected seeds to non-infected 'Knuckle Purple Hull' plants. Two aphids (M. persicae) were used per test plant and each aphid was allowed to have an acquisition period of 30 to 60 sec. A single test plant showing typical mosaic was assayed by leaf-dip electron microscopy for the presence of

rod-shaped virus particles and used as the initial source of inoculum for virus propagation. The virus was mechanically transmitted from the selected infected plant to healthy 'Knuckle Purple Hull' seedlings, where it was increased for virus and inclusion purification, and other studies.

Virus and Inclusion Purification

Blackeye cowpea mosaic virus was propagated in either V. unguiculata or Nicotiana benthamiana Domin, and systemically infected leaves were used for virus and inclusion purification. Either n-butanol or a combination of chloroform and carbon tetrachloride was used in the clarification process. The adaxial surface of the primary leaves of 5 to 7-day old cowpea seedlings were inoculated with BICMV obtained by grinding infected leaf tissue in 0.05 M potassium phosphate (KPO_4) buffer, pH 7.5 (1/2, w/v). The first trifoliolate leaves showing typical mosaic were collected 15 to 18 days later and subjected to the following purification procedures based on previous works (Hiebert et al., 1971; Hiebert and McDonald, 1973; and McDonald and Hiebert, 1975).

n Butanol clarification method. Two hundred to 400 g of leaf tissue were homogenized in a blender with two parts (w/v) of 0.5 M KPO_4 buffer, pH 7.5, containing 0.5 to 1.0% sodium sulfite (Na_2SO_3). The resulting extract was filtered through a double layer of cheesecloth and enough n-butanol was added to make a final concentration of 8% (v/v). This mixture was stirred overnight at 4 C and the coagulated green debris obtained was removed by a low speed centrifugation at 11,700 g in a Sorvall Centrifuge (Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge) for 10 min. Virions were precipitated from the supernantant by the addition of 6 - 8% (w/v) of polyethylene glycol

MW 6000 (PEG) followed by stirring for 60 min. The precipitated virions were collected by centrifugation at 13,200 g for 10 min. The resulting pellet was resuspended in 0.02 M KPO_4, pH 8.2, containing 0.1% 2-mercapthoethanol (2-ME) (v/v) and the virus was separated from the host components by equilibrium density gradient centrifugation (120,000 g for 16 - 18 hr in a Beckman SW 50.1 rotor) in 30% cesium chloride (CsCl) prepared in the same buffer. The virus zone, located at 12 to 15 mm from the bottom of the tube, was collected dropwise through a hole punched in the bottom of the tube and diluted with 0.02 M KPO_4, pH 8.2, containing 0.1% 2-ME. The virus preparation was clarified by centrifugation at 11,700 g for 10 min and reconcentrated by centrifugation at 85,000 g for 90 min. The final pellet was resuspended in 0.02 M Tris buffer, pH 8.2, and the virus concentration was determined spectrophotometrically using an extinction coefficient of 2.4 mg/ml (Purcifull, 1966). The optical density (O.D.) readings for the virus at wavelengths of 260 and 280 nm were corrected for light scattering before estimating the 260/280 ratio and concentrations of virus in purified preparations. The correction for light scattering was done by plotting the log of the optical densities against the wavelengths of 320, 340, and 360 nm and extrapolating these values to 230 - 300 nm range of wavelength.

Chloroform-carbon tetrachloride clarification method. This clarification process was selected when it was desirable to purify both the virus and inclusions from the same batch of tissue. Systemically infected tissue (200 - 400 g) were homogenized in a solution containing 1.30 ml of 0.5 M KPO_4 (pH 7.5), 0.35 ml of chloroform, 0.35 ml of carbon tetrachloride, and 5.0 mg of Na_2SO_3 per gram of tissue.

The homogenized mixture was centrifuged in a Sorvall Centrifuge at 5,000 rpm for 5 min and the pellet containing the organic solvents was discarded. The aqueous phase was centrifuged at 13,200 g for 15 min to precipitate the virus induced inclusions. The supernatant was treated as previously described for virus purification and the pellet containing the inclusions was resuspended in 0.05 M KPO_4, pH 8.2, and 0.1% 2-ME. The inclusion suspension was homogenized in a Sorvall Omni-mixer homogenizer for 2 min and enough Triton X-100 was added to make a final concentration of 5% (v/v). After stirring for one hour at 4 C this mixture was subjected to a low speed centrifugation of 27,000 g for 15 min to precipitate the inclusions. The pellet was resuspended in 10 to 20 ml of 0.02 M KPO_4, pH 8.2, containing 0.1% 2-ME, and homogenized for 30 sec. The inclusions were sedimented again by centrifugation of 27,000 g for 15 min. The pellet was homogenized for 30 sec and the homogenate was layered on a sucrose step gradient made up of 10 ml of 80%, 7 ml of 60%, and 7 ml of 50% (w/v) sucrose in 0.02 M KPO_4, pH 8.2. The gradient was centrifuged for one hour at 27,000 rpm in a Beckman SW 25.1 rotor. The inclusions layered on top of the 80% sucrose zone and were collected by droplet from the bottom of the tube. To remove the sucrose, the inclusions were diluted in 0.02 M KPO_4, pH 8.2, and precipitated by a centrifugation at 27,000 g for 15 min. The pellet was resuspended in 0.02 M Tris, pH 8.2, and inclusion yield was estimated spectrophotometrically after being disrupted in 2% sodium dodecyl sulfate (SDS). The inclusion preparations were either immediately used for immunization of rabbits or stored at -20 C by either freezing directly or by freeze-drying.

Clarification with n-butanol and chloroform-carbon tetrachloride.

Because n-butanol resulted in virus preparations of higher purity, but chloroform-carbon tetrachloride was superior for preservation of inclusion proteins (Hiebert, unpublished), these solvent systems were combined for purification of virus and inclusions from the same batch of tissue. Infected tissue was homogenized with two parts (w/v) of 0.5 M KPO_4, pH 7.5, containing 0.5 - 1.0% Na_2SO_3. The homogenate was filtered through cheesecloth and subjected to centrifugation at 11,700 g for 10 min. The supernatant was used for virus purification as described previously using n-butanol for clarification. The pellet was resuspended in approximately 2 volumes of 0.5 M KPO_4, pH 8.2, 0.5% Na_2SO_3, homogenized with one volume of chloroform-carbon tetrachloride (1:1, v/v) and centrifuged at 5,000 rpm for 5 min in a Sorvall Centrifuge. The aqueous phase was subjected to a centrifugation at 11,700 g for 15 min. The supernatant was collected for additional virus purification using PEG, equilibrium density-gradient centrifugation and differential centrifugation. The pellet was resuspended in 0.05 M KPO_4, pH 8.2, containing 0.1% 2-ME and treated with 5% Triton X-100. The inclusions were then purified by sucrose step gradient centrifugation as described above.

Virus Particle Size Determination

Crude leaf extracts from systemically infected cowpea plants and purified virus preparations were negatively stained in 2% potassium phosphotungstate (PTA), pH 6.5, containing 0.1% bovine serum albumin (BSA) prior to photography in an electron microscope. The procedure used was similar to those previously described (Edwardson et al., 1968 and Purcifull et al., 1970). Small pieces of BlCMV-infected leaf were

chopped with a razor blade in 2% PTA, pH 6.5, containing 0.1% BSA on a glass slide and a small quantity of the resulting cell extract was deposited on a carbon coated Formvar film supported by 75 x 300 mesh copper grids. Excess liquid was then removed by touching momentarily the edge of the grid with a filter paper and the specimen was allowed to air-dry. The purified virus was stained directly on the grid. A small drop of virus solution was deposited on the grid. After 1 - 2 min, the virus solution was partially blotted with a piece of filter paper and a small drop of 2% PTA solution was added. The grid was blotted and allowed to air-dry. The grids were then examined in a Philips Model 200 electron microscope. The virus particles were observed, photographed and their sizes were estimated by comparing projected micrographs to micrographs of a diffraction grating (2160 lines/mm). Twenty-five virus particles from leaf extracts and 190 particles from a purified preparation were measured and classified according to their length at intervals of 50 and 20 nm.

Blackeye cowpea mosaic virus-grids prepared according to the serologically specific electron microscopic technique (SSEM) developed by Derrick and Brlansky (1976) were also used for virus particle measurements. Parlodion film grids sensitized with BICMV-antiserum (BICMV-As) were treated with cowpea leaf extract containing BICMV and positively stained with 1% uranyl acetate in 50% ethanol. The SSEM technique will be described in more detail in Chapter II.

Stability of Virus in Sap

Thermal inactivation point (TIP), longevity in vitro (LIV), and dilution end point (DEP) were determined for BICMV using C. amaranticolor as an assay plant. The TIP was determined by heating crude sap of

BICMV-infected cowpea leaves to 45, 50, 55, 60, 65, 70, and 75 C for 10 min. All treated saps as well as unheated sap of BICMV-infected tissue were rubbed on the test plants, which were maintained in greenhouse conditions for at least three weeks for observation of symptoms.

Crude sap of infected leaves obtained in deionized water was placed in test tubes and assayed for infectivity after storage at room temperature for 0, 8, 16, 24, 48, and 72 hr. For the DEP determination, crude juice was extracted from BICMV-infected leaves, and the extract was diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} with deionized water prior to assay.

Polyacrylamide Gel Electrophoresis of Viral and Inclusion Proteins

The polyacrylamide gel electrophoresis studies were performed according to the method of Weber and Osborn (1969) as modified by Hiebert and McDonald (1973). Running gels of approximately 75 mm in height were prepared with 6% acrylamide (7.5 ml sodium phosphate buffer, pH 7.2; 15.0 ml water; 0.15 ml 10% SDS; 6.0 ml of 30% acrylamide; 0.045 ml N, N, N', N'-tetramethylenediamine (TEMED) and 1.2 ml ammonium persulphate 15 mg/ml), and a well-forming gel of 8% acrylamide with one-fifth the electrophoresis buffer concentration (1.2 ml buffer; 7.2 ml H_2O; 0.2 ml 10% SDS; 3.0 ml of 30% acrylamide; 0.04 ml TEMED, and 0.3 ml ammonium persulphate 15 mg/ml) was cast on top of them. Disassociated protein solutions, 20 - 50 µl samples in approximately 20% sucrose and one-fifth the electrophoresis buffer concentration, were placed into the formed wells. The top of the samples were covered with a cap gel of composition similar to the well-forming gel. The electrophoresis was performed in a vertical slab electrophoresis apparatus, Ortec, Model 4010/4011, Ortec, Incorporated, Oak Ridge, Tenn.,

for 1.5 to 4.0 hr at 160 V with a pulsed constant power supply at 300 pulses per second and about 90 mA current.

Prior to being used for electrophoresis, the protein was disassociated by mixing 0.2 ml of protein solution with 0.1 ml of 10% SDS and 10 - 20 µl 2-ME and heating this mixture in boiling water for 1 to 2 min. Samples of 20 - 50 µl of disassociated proteins were added to 0.1 ml of one-fifth of the electrophoresis buffer concentration, containing 30% sucrose and 0.15% SDS.

Serum albumin (MW 67,000), glutamate dehydrogenase (MW 53,000), ovalbumin (MW 43,000), carbonic anhydrase (MW 29,000), and TMV coat protein (MW 17,500) were used as protein markers to estimate the molecular weight values for inclusions and virus coat protein subunits.

After electrophoresis, the gel slabs were stained and fixed overnight in a staining solution containing 50% methanol, 10% glacial acetic acid, and 0.1% Coomassie brilliant blue R250. Before photography, the gels were destained by soaking them for 8 hr in a solution made up of 10% methanol and 7.5% acetic acid followed by several changes in the solution over a period of several days. The distances migrated by the protein subunits into the running gels were measured from the photographs of the stained gels.

Sedimentation Coefficient Determination

The sedimentation rates of fresh and stored purified BlCMV in either 0.02 M Tris buffer, pH 8.2 or 0.05 M borate buffer, pH 8.2 were measured with a Beckman Model E analytical ultracentrifuge according to the method of Markham (1960). After the rotor reached a speed of 27,690 rpm photographs were taken at 4 min intervals using

Schlieren optics. The data were corrected for standard water viscosity conditions at 20 C, but not for the effect of virus concentration. The virus concentrations used varied from 0.5 to 1.0 mg/ml.

Serology

Antiserum production for virus and cytoplasmic inclusions. Antisera were obtained by injecting a New Zealand white rabbit with untreated virions and a second rabbit with pyrrolidine-degraded virus protein. All rabbits selected for immunization were first bled to produce normal sera. The concentrations of untreated BICMV in 0.02 M Tris buffer, pH 8.2, used in the immunization process varied from 1.0 to 2.0 mg of nucleoprotein per ml of purified solution. BICMV used for pyrrolidine degradation was suspended in 0.005 M borate buffer, pH 8.2. The virus protein was degraded according to the method used by Shepard (1972). A virus solution was mixed with an equal volume of 5% pyrrolidine in distilled water (v/v). The mixture was then immediately dialyzed against two liters of 0.05 M borate buffer, pH 8.2, containing 0.37% actual formaldehyde for approximately 48 hr at 4 C to remove the pyrrolidine and fix the protein subunits.

A series of 4 to 5 intramuscular injections was given to each rabbit with an interval of 10 to 15 days between the injections. Each injection consisted of 1.0 to 2.0 ml preparations of virus or degraded viral protein vigorously emulsified with equal volume of Freund's complete or incomplete adjuvants (Difco). Booster injections were given at intervals of about 2 months.

The immunized animals were bled every week, starting 10 to 15 days after the last injection of the initial series of 4 - 5 injections.

The rabbits were fasted for 4 - 12 hr prior to each bleeding and 30 - 50 ml of blood were collected into glass tubes according to the procedure described by Purcifull and Batchelor (1977). Blood samples were allowed to clot for approximately 45 min at 37 C in a waterbath. The clotted blood was subjected to a centrifugation of 2,000 rpm in a Sorvall table centrifuge for 10 min. The antisera were transferred with a Pasteur pipette to conical-bottomed tubes and clarified by a second centrifugation at 5,000 rpm for 10 min. Antiserum specificity and titer were determined by Ouchterlony (1962) double-diffusion tests in SDS-agar plates. The antisera were stored at -20 C by either freezing directly or after freeze-drying.

The BICMV-induced cytoplasmic inclusions (BICMV-I) used for antiserum production were purified from N. benthamiana. Freshly purified cylindrical inclusions, which were unreactive with antiviral sera, were used for immunization and the foot pad route of immunization (Ziemiecki and Wood, 1975) was used. The rabbit received three injections into the foot pad, each containing 0.1 ml of purified inclusions (0.1 - 0.2 O.D. units/ml at 280 nm) in 0.02 M Tris, pH 8.2, emulsified with an equal volume of either Freund's complete or incomplete adjuvants.

Serological tests. Both double and single immunodiffusion tests in agar gel were used in the present study. Most double immunodiffusion tests were performed in agar medium containing 0.8% Noble agar (Difco); 0.5% SDS (Sigma) and 1.0% NaN_3 (Sigma) in deionized water (Purcifull and Batchelor, 1977), or 0.05 M Tris-HCl buffer pH 7.2. Reactant wells were punched in the solidified agar medium with an adjustable gel cutting device made by Grafar Corp., Detroit, Mich. Routinely the wells (7 mm in diameter) were punched in an hexagonal arrangement

consisting of a center well with six peripheral wells spaced 4 - 5 mm from the center well as measured from the edges of the wells. Different gel patterns were also used in certain tests. Antigens used as reactants were prepared either in deionized water or in 1.5% SDS solution, according to Purcifull and Batchelor (1977). In the first case, fresh tissue was ground with a mortar and pestle in deionized water (1/2, w/v) and expressed through cheesecloth. The second method which was more commonly used, consisted of grinding fresh tissue in 1.0 ml of water per gram of tissue and adding 1.0 ml of 3.0% SDS per gram of tissue prior to expressing the sap through cheesecloth. The antigens and undiluted antisera were pipetted directly into the appropriate wells, and the plates were incubated in a moist chamber at 24 C for 24 - 48 hr. The development of precipitation patterns was observed by looking at the plates, which were illuminated from the bottom with indirect lighting. The reactants were removed and 15% charcoal (Norit A) in water (w/v) was added into the wells before photographs were taken.

Single radial immunodiffusion tests were conducted in agar media containing 0.8% Noble agar, 1.0% NaN_3, 0.3 or 0.5% SDS, and 10, 15, or 20% BICMV antiserum. Media were prepared either with antiserum obtained for untreated BICMV and antiserum for pyrrolidine degraded BICMV-protein. Each SDS concentration in the media was tested with antigens prepared in distilled water or in 1.5% SDS. During medium preparation, care was taken to avoid heating the antisera over 50 C and while exposed to SDS, the antisera were maintained at 50 C for less than 2 min.

Single radial diffusion plates were also prepared with a mixture of antisera to BICMV and CPMV. The CPMV-antiserum was prepared by

immunizing a rabbit with CPMV degraded by SDS according to a procedure described by Purcifull and Batchelor (1977). A lyophilized, purified preparation containing approximately 3 mg of CPMV was resuspended in 1 ml of 1.0% SDS solution containing 2.0% 2-ME, and boiled for approximately 5 min before emulsification with Freund's adjuvant and intramuscular injection into a rabbit. Three similar injections were given into the same rabbit with 7-day intervals between injections.

Serological relationship between BlCMV and other potyviruses. Reciprocal double immunodiffusion tests with BlCMV and the following potyviruses were conducted in SDS-containing media: bean yellow mosaic virus (BYMV), bean common mosaic virus (BCMV-BV-1), bean common mosaic virus-siratro isolate (BCMV-S), bidens mottle virus (BiMV), dasheen mosaic virus (DMV), lettuce mosaic virus (LMV), pepper mottle virus (PeMV), potato virus Y (PVY), soybean mosaic virus (SoyMV), tobacco etch virus (TEV), turnip mosaic virus (TuMV), watermelon mosaic virus-1 (WMV-1), and watermelon mosaic virus-2 (WMV-2). The source of each antiserum was as follows: BYMV (Jones and Diachun, 1977); BCMV-BV-1 (J. K. Uyemoto, New York State Agricultural Experiment Station); BCMV-S (Lima et al., 1977); DMV (Abo El-Nil et al., 1977); BiMV, LMV, PeMV, PVY, SoyMV, TEV, TuMV, WMV-1, and WMV-2 (D. E. Purcifull, University of Florida, Gainesville).

Using BlCMV-As, the serological relationship of BlCMV with commelina mosaic virus (CoMV) (Morales and Zettler, 1977), a Moroccan isolate of CAMV (Fischer and Lockhart, 1976a), pepper veinal mottle virus (PVMV) and pokeweed mosaic virus (PWMV) were also studied in double diffusion tests with SDS-treated antigens. In all serological tests, the reactants were arranged so that BlCMV was always placed in

a well adjacent to the other virus-well. Sap extracts from appropriate healthy host tissues were included as controls in all serological tests, and all antigens were also tested against normal serum.

The intragel cross-absorption technique described by van Regenmortel (1966) was also used to study the serological relationships of BlCMV with BCMV-S and CAMV. Purified preparations of heterologous antigens (BCMV-S or CAMV) were placed in the center well and allowed to diffuse for approximately 24 hr. The excess of the antigen preparations were then removed and the BlCMV antiserum was added in the same well. At the same time, the homologous and the heterologous antigens were positioned in the outer wells.

Light and Electron Microscopy of Virus Induced Pinwheel Inclusions

Epidermal leaf strips obtained from systemically infected cowpea, V. unguiculata, were floated on a 5% solution of Triton X-100 for 5 to 10 min and subsequently stained with a combination of calcomine orange and "luxol" brilliant green as described by Christie (1967). The stained leaf strips were mounted in euparal on glass slides and examined with a light microscope for the presence of cytoplasmic inclusions. Similarly, strips from noninoculated V. unguiculata were also stained and examined in the light microscope as controls.

Cylindrical inclusions were examined in situ in ultrathin sections with an electron microscope. Small pieces were taken from symptomatic areas of systemically infected cowpea leaves and fixed for 2 to 3 hr at room temperature in Karnovsky's formaldehyde-glutaraldehyde fixative prepared in 0.1 M cacodylate buffer, pH 7.2 (Karnovsky, 1965). After washing with 0.1 M cacodylate buffer, the small leaf pieces were postfixed for 1 to 2 hr at room temperature

in 2% osmium tetroxide and progressively dehydrated in an increasing ethanol solution series. The leaf pieces were maintained for 5 to 15 min in each ethanol solution at room temperature. The pieces were stained overnight at 4 C in a solution of 75% ethanol containing 2% uranyl acetate and subsequently dehydrated in a second series of ethanol solutions (75 - 100%) followed by 100% acetone or propylene oxide. They were then embedded in plastic containing Epon 812, Araldite 502, and dodecenylsuccinic anhydride. Ultrathin sections were cut with a diamond knife in a Sorvall MT-2 ultramicrotome and mounted on copper grids with carbon-coated Formvar film. The specimens mounted on the grids were poststained with 9% potassium permanganate (2 min), 1% uranyl acetate (2 min), and lead citrate (2 min). These sections as well as those obtained from noninoculated cowpea plants were examined with a Philips Model 200 electron microscope.

Purified BlCMV-I preparations were mounted on carbon-coated Formvar film supported by copper grids and stained with either 1% ammonium molybdate or 2% uranyl acetate, before examination by electron microscopy.

Host Range and Screening Cowpea Varieties for Resistance

Test plants were inoculated with crude sap from 'Knuckle Purple Hull' systemically infected with BlCMV. The inoculum was prepared by grinding leaf tissue in 0.05 M KPO_4, pH 7.5 (1/2, w/v). The inoculations were done by rubbing the inoculum on carborundum-dusted leaves of the test plants which were maintained in greenhouse conditions for at least one month for observation of symptoms. All inoculated plants, including those that did not show any symptoms were checked serologically for the presence of BlCMV.

The cowpea varieties were also inoculated with CPMV, CAMV, and BCMV-S. Crude sap from all inoculated cowpea plants were also tested in double immunodiffusion against antisera specific for CPMV, BlCMV, and BCMV-S, respectively. Since CAMV was shown to be serologically related to BlCMV, the serological tests to detect its presence in the inoculated plants were done with BlCMV antiserum.

Results

Purification and Properties of Blackeye Cowpea Mosaic Virus

Purified preparations of BlCMV were obtained from systemically infected leaves of either V. unguiculata 'Knuckle Purple Hull' (Fig. 1-A) or N. benthamiana using the purification procedures diagrammed in Figures 2, 3, and 4. The best yield with the highest degree of purity was obtained using the first method of virus purification (Fig. 2) and infected cowpea leaves (Fig. 1-A) as a source of virus. The first trifoliolate cowpea leaves collected 15 to 18 days after inoculations gave the highest yield of virus (8 - 10 mg) per 100 g (fresh weight) of infected tissue and n-butanol proved to be the best clarifying agent for cowpea tissue. An opalescent, sharp virus-band was usually obtained after equilibrium density gradient centrifugation in 30% CsCl. The virus zone was located at 12 to 15 nm from the bottom of the tube while most of the green host components stayed at the top portion of the gradient. The clear pellet obtained after a high speed centrifugation of virus removed from CsCl gradients confirmed the absence of colored host components. The combination of chloroform and carbon tetrachloride, although necessary for inclusion purification, was an inferior method of clarification for obtaining virus from cowpea

Figure 1 - Systemic and localized symptoms induced by blackeye cowpea mosaic virus (BlCMV) in cowpea, V. unguiculata 'Knuckle Purple Hull' and C. amaranticolor.

A) Typical mosaic on secondary trifoliolate leaf of cowpea plant inoculated with BlCMV (1), and primary trifoliate leaf showing vein clearing (2).

B) Local lesions on leaf of C. amaranticolor inoculated with BlCMV.

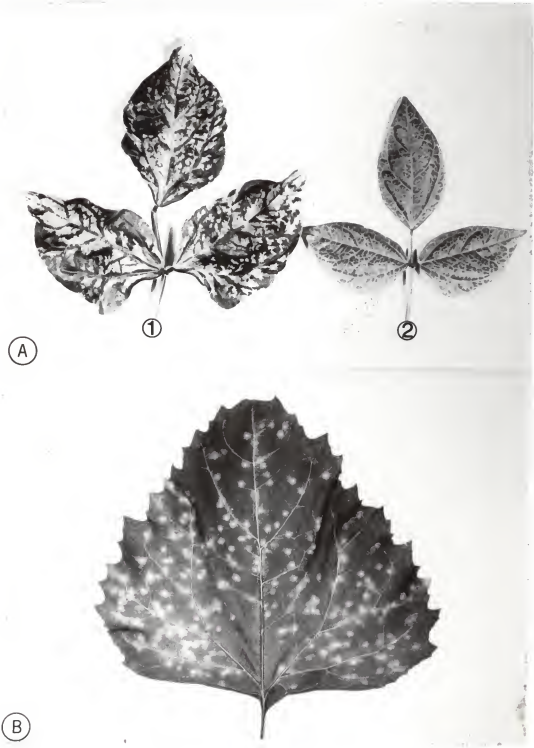

A
①
②
B

Figure 2 - Flow diagram outlining the procedure of purification of BlCMV using n-butanol as clarifying agent, polyethylene glycol (PEG) for virus concentration, CsCl gradient centrifugation for separation of virus from host components, and differential centrifugation for further virus purification. For details, see description in materials and methods section.

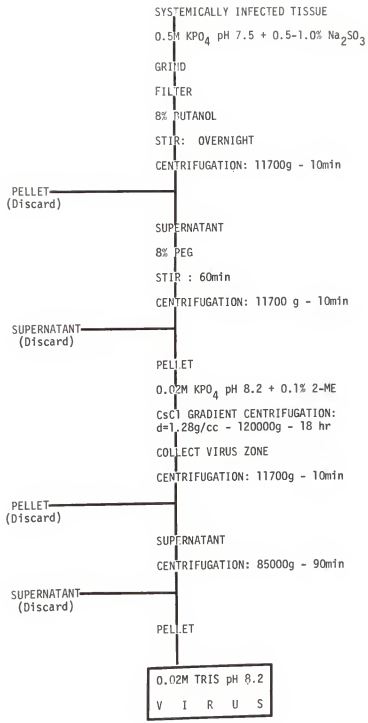

SYSTEMICALLY INFECTED TISSUE
0.5M KPO_4 pH 7.5 + 0.5-1.0% Na_2SO_3
GRIND
FILTER
8% BUTANOL
STIR: OVERNIGHT
CENTRIFUGATION: 11700g - 10min
PELLET
(Discard)
SUPERNATANT
8% PEG
STIR : 60min
CENTRIFUGATION: 11700 g - 10min
SUPERNATANT
(Discard)
PELLET
0.02M KPO_4 pH 8.2 + 0.1% 2-ME
CsCl GRADIENT CENTRIFUGATION:
d=1.28g/cc - 120000g - 18 hr
COLLECT VIRUS ZONE
CENTRIFUGATION: 11700g - 10min
PELLET
(Discard)
SUPERNATANT
CENTRIFUGATION: 85000g - 90min
SUPERNATANT
(Discard)
PELLET
0.02M TRIS pH 8.2
V I R U S

Figure 3 - Flow diagram outlining the procedure for purification of BlCMV and its cytoplasmic inclusions, using chloroform and carbon tetrachloride as clarifying agents. The procedure is described in the text.

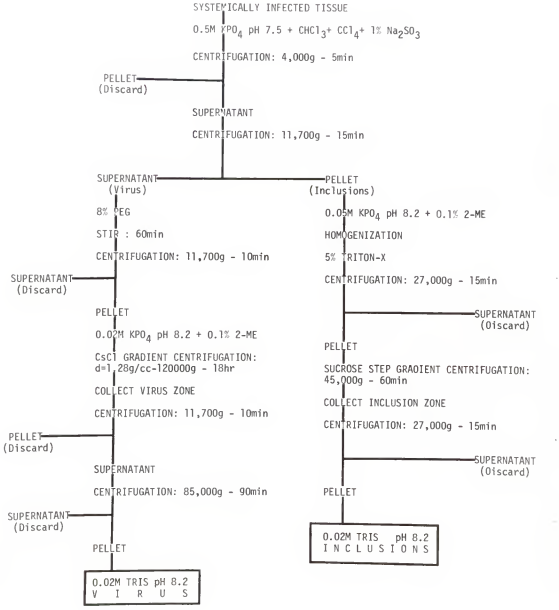
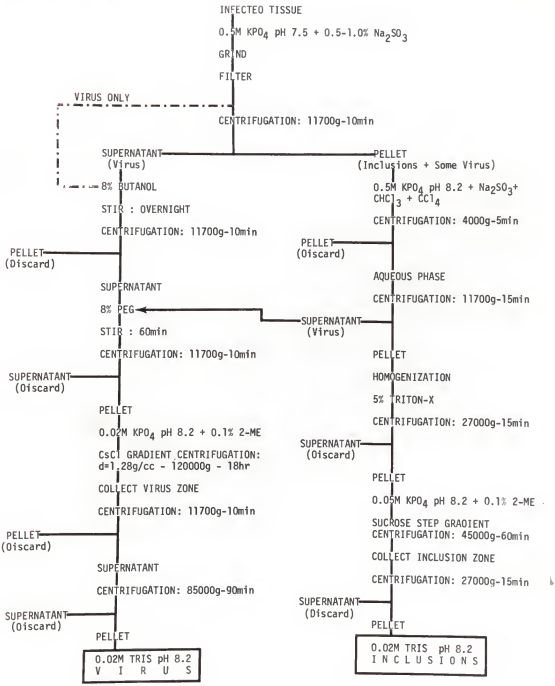

SYSTEMICALLY INFECTED TISSUE
0.5M KPO4 pH 7.5 + CHCl3+ CCl4+ 1% Na2SO3
CENTRIFUGATION: 4,000g - 5min
PELLET (Discard)
SUPERNATANT
CENTRIFUGATION: 11,700g - 15min
SUPERNATANT (Virus)
8% PEG
STIR : 60min
CENTRIFUGATION: 11,700g - 10min
SUPERNATANT (Discard)
PELLET
0.02M KPO4 pH 8.2 + 0.1% 2-ME
CsCl GRADIENT CENTRIFUGATION: d=1.28g/cc-120000g - 18hr
COLLECT VIRUS ZONE
CENTRIFUGATION: 11,700g - 10min
PELLET (Discard)
SUPERNATANT
CENTRIFUGATION: 85,000g - 90min
SUPERNATANT (Discard)
PELLET
0.02M TRIS pH 8.2 V I R U S
PELLET (Inclusions)
0.05M KPO4 pH 8.2 + 0.1% 2-ME
HOMOGENIZATION
5% TRITON-X
CENTRIFUGATION: 27,000g - 15min
SUPERNATANT (Discard)
PELLET
SUCROSE STEP GRADIENT CENTRIFUGATION: 45,000g - 60min
COLLECT INCLUSION ZONE
CENTRIFUGATION: 27,000g - 15min
SUPERNATANT (Discard)
PELLET
0.02M TRIS pH 8.2 I N C L U S I O N S

Figure 4 - Flow diagram outlining the steps carried out during the purification of BlCMV and its cytoplasmic inclusions by a combination of the first (Fig. 2) and second (Fig. 3) methods for purification of virus and inclusions.


INFECTED TISSUE
0.5M KPO4 pH 7.5 + 0.5-1.0% Na2SO3
GRIND
FILTER
VIRUS ONLY
CENTRIFUGATION: 11700g-10min
SUPERNATANT (Virus)
PELLET (Inclusions + Some Virus)
8% BUTANOL
STIR : OVERNIGHT
CENTRIFUGATION: 11700g-10min
PELLET (Discard)
SUPERNATANT
8% PEG
STIR : 60min
CENTRIFUGATION: 11700g-10min
SUPERNATANT (Discard)
PELLET
0.02M KPO4 pH 8.2 + 0.1% 2-ME
CsCl GRADIENT CENTRIFUGATION: d=1.28g/cc - 120000g - 18hr
COLLECT VIRUS ZONE
CENTRIFUGATION: 11700g-10min
PELLET (Discard)
SUPERNATANT
CENTRIFUGATION: 85000g-90min
SUPERNATANT (Discard)
PELLET
0.02M TRIS pH 8.2
V I R U S
0.5M KPO4 pH 8.2 + Na2SO3+ CHCl3 + CCl4
CENTRIFUGATION: 4000g-5min
PELLET (Discard)
AQUEOUS PHASE
CENTRIFUGATION: 11700g-15min
SUPERNATANT (Virus)
PELLET
HOMOGENIZATION
5% TRITON-X
CENTRIFUGATION: 27000g-15min
SUPERNATANT (Discard)
PELLET
0.05M KPO4 pH 8.2 + 0.1% 2-ME
SUCROSE STEP GRADIENT CENTRIFUGATION: 45000g-60min
COLLECT INCLUSION ZONE
CENTRIFUGATION: 27000g-15min
SUPERNATANT (Discard)
PELLET
0.02M TRIS pH 8.2
I N C L U S I O N S

tissues. With this method, a clear sap was obtained after the first low speed centrifugation but the virus zone in the CsCl gradient was not very well separated from the host components.

Plants of C. amaranticolor and V. unguiculata mechanically inoculated with purified preparations of BlCMV showed the first symptoms of local lesions and systemic mosaic (Fig. 1) 4 and 7 days after inoculation, respectively. The ultraviolet absorption curve (Fig. 5) obtained for the purified preparations of BlCMV had a maximum between 260 and 262 nm, and a minimum at 244 to 245 nm. The ratio between the absorption at wavelengths of 260 and 280 nm was approximately 1.2 after correction for light scattering, as would be expected for a member of the PVY group. This value is consistent and agrees with those of other long flexuous rod-shaped viruses (Shepherd and Purcifull, 1971; Tosic et al., 1974; and Barnett and Alper, 1977). The virus solutions showed strong stream birefringence and electron microscopic examinations indicated that 73% of the 190 virus particles examined were between 700 and 800 nm (Figs. 6, 7, 8). The rods observed in the purified preparations (Fig. 6) indicated a low percentage of virus fragmentation during the purification processes. As the result of end-to-end virus aggregation, a few particles with 1400 to 1500 nm were also observed. Purified virus preparations usually were relatively free of normal plant constituents when examined with the electron microscope and in the spectrophotometer.

Sedimentation coefficients determined for the virus at 20 C either in 0.02 M Tris buffer, pH 8.2, or in 0.05 M borate buffer, pH 8.2, indicated that BlCMV sedimented as a single species with the s_{20} values of 157 - 159 S. On the other hand, the Schlieren pattern (Fig. 9)

Figure 5 - Absorption spectra of purified preparations of BlCMV in 0.02 M Tris buffer, pH 8.2, and BlCMV cytoplasmic inclusions in the same buffer.

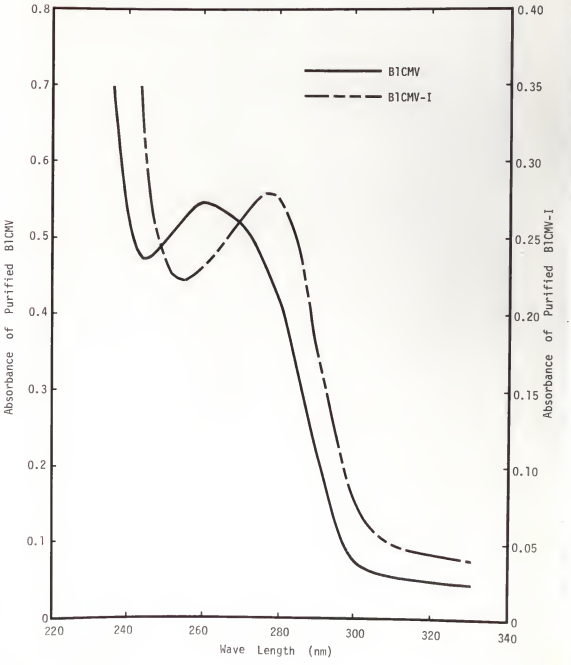

BlCMV
BlCMV-I
Absorbance of Purified BlCMV
Absorbance of Purified BlCMV-I
Wave Length (nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
220
240
260
280
300
320
340

Figure 6 - Electron microscopy of BlCMV in a purified preparation and in cowpea leaf extracts.

A) Purified preparation of BlCMV negatively stained with 2% phosphotungstic acid, pH 6.5, containing 0.1% BSA;

B) Serologically specific electron microscopy (SSEM) of leaf extract from cowpea plants systemically infected with BlCMV. Antiserum for BlCMV diluted 1/1000 in 0.05 M Tris buffer, pH 7.2, was used to sensitize the grid and the virus particles were positively stained with 1% uranyl acetate. Note the considerable increase in virus concentration compared with the normal leaf-dip preparation (C);

C) Leaf-dip preparation of cowpea leaf tissue systemically infected with BlCMV, negatively stained with 2% phosphotungstate.

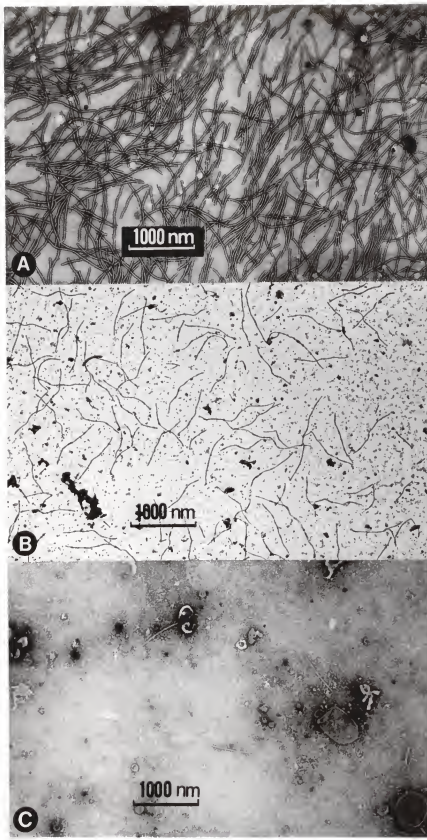

1000 nm
A
1000 nm
B
1000 nm
C

Figure 7 - Histograms of lengths of BICMV particles from purified preparation negatively stained with phosphotungstate (A), and cowpea leaf extract using the serologically specific electron microscopy and uranyl acetate as a positive stain (B). Class interval for both histograms = 50 nm.

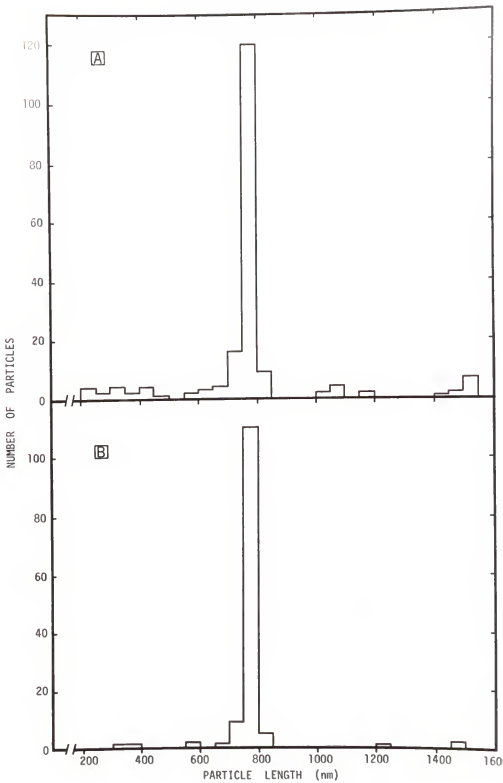
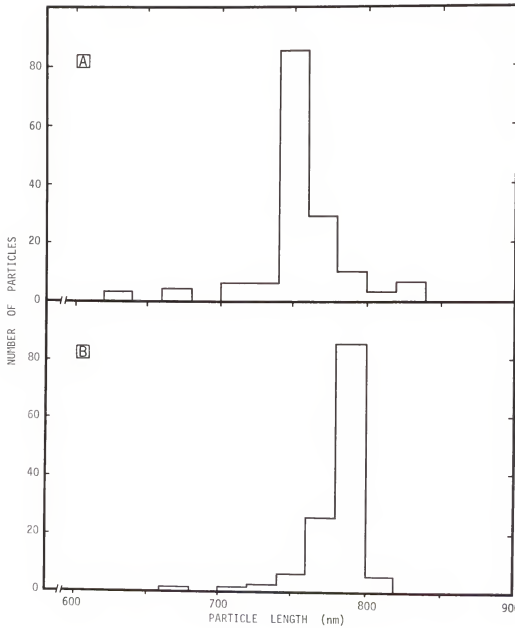

A
B
120
100
80
60
40
20
0
100
80
60
40
20
0
NUMBER OF PARTICLES
200
400
600
800
1000
1200
1400
1600
PARTICLE LENGTH (nm)

Figure 8 - Histograms of BlCMV particle lengths from two different electron microscopic preparations to show particle length distribution from 600 to 900 nm. Class interval = 20 nm.

A) Particle length distribution of BlCMV from purified preparation negatively stained with phosphotungstate;

B) Particle length distribution of BlCMV from cowpea leaf extract prepared on grids sensitized with BlCMV antiserum and positively stained with uranyl acetate.


A
B
80
60
40
20
0
NUMBER OF PARTICLES
600
700
800
900
PARTICLE LENGTH (nm)

revealed a difference in S values between BlCMV in fresh preparations and BlCMV in purified preparations stored at 4 C for more than 30 days. Both virus preparations showed a single sedimenting peak, but the s_{20} values for BlCMV in fresh preparations and at a concentration varying from 0.5 to 1.0 mg/ml ranged from 157 to 159 S while the s_{20} values for the virus in the stored preparations and at the same concentrations ranged from 140 to 142 S (Fig. 9). The lower sedimentation coefficients obtained for the stored virus suggested that a change in virus mass (MW) had occurred. Hiebert and McDonald (1976) observed some possible enzymatic degradation of capsid protein of purified turnip mosaic virus. Proteolytic degradation of capsid protein of stored purified preparations of BlCMV was also observed by polyacrylamide gel electrophoresis (PAGE) studies. Polyacrylamide gel electrophoresis analysis of SDS-degraded virus of a freshly purified preparation of BlCMV revealed a main protein component with an estimated molecular weight (MW) of 34,000 daltons and two smaller ones with MWs of 29,000 and 27,000 daltons (Fig. 10). These smaller components may have arisen by degradation of the slow moving component during storage (Hiebert and McDonald, 1976). Stored BlCMV preparations contained only the faster moving protein components with MWs of 29,000 and 27,000 daltons (Fig. 10), presumably derived from 34,000 daltons component.

Purified Inclusion Preparations

Using either of the methods outlined in Figs. 3 and 4, purified cylindrical inclusions induced by BlCMV were obtained from the same batches of systemically infected leaf tissue of *V. unguiculata* or

Figure 9 - Schlieren patterns from sedimentation velocity experiment with stored (A), and fresh (B), purified preparations of BICMV. Photograph was taken 8 minutes after the rotor reached a speed of 27,690 rpm. Sedimentation is from left to right.

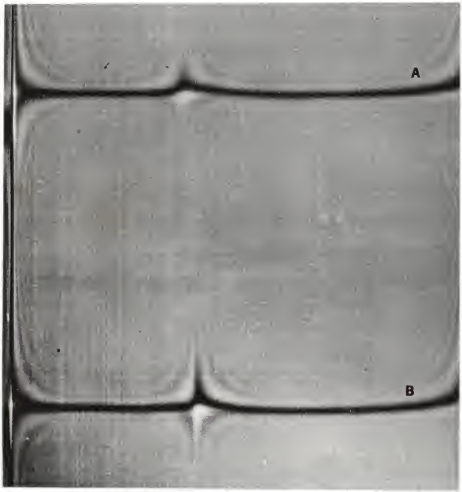
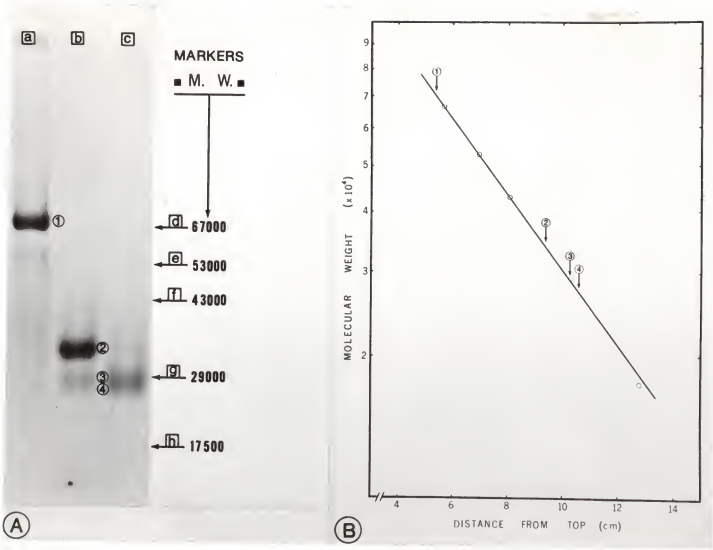

A
B

Figure 10 - Electrophoretic analyses of B1CMV induced cytoplasmic inclusions (B1CMV-I) and B1CMV capsid protein in a 6% polyacrylamide gel slab containing 0.1% sodium dodecyl sulfate (SDS) and sodium phosphate buffer, pH 7.2.

A) Polyacrylamide gel electrophoresis of SDS dissociated proteins of B1CMV-I (a), viral coat protein of freshly purified preparation of B1CMV (b), viral coat protein of purified B1CMV after 30 days of storage at 4 C, (c), and marker proteins (d-h). The migrations of the marker proteins on the gel are represented by the arrows: (d) bovine serum albumin, (e) glutamate dehydrogenase, (f) ovalbumin, (g) carbonic anhydrase, and (h) TMV capsid protein.

B) Molecular weight determination of SDS dissociated proteins of B1CMV-I and capsid protein of fresh and old purified B1CMV preparations in polyacrylamide gel electrophoresis using marker proteins. The unnumbered circles along the standard line represent the following marker proteins with their molecular weights in parentheses from top to bottom: bovine serum albumin (67,000 daltons), glutamate dehydrogenase (53,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (29,000 daltons), and TMV capsid protein (17,500 daltons). The distances from the top, correspond to the distances measured in photograph positioned as in (A). The estimated molecular weights for the virus and its cytoplasmic inclusion proteins are as follows: (1) 70,000 daltons (B1CMV-I), (2) 34,000 daltons (undegraded B1CMV coat protein), (3) 29,000 daltons (degraded B1CMV coat protein), and (4) 27,000 daltons (degraded virus protein).


a
b
c
MARKERS
M. W.
d 67000
e 53000
f 43000
g 29000
h 17500
①
②
③
④
A
MOLECULAR WEIGHT (x10⁴)
9
8
7
6
5
4
3
2
4
6
8
10
12
14
DISTANCE FROM TOP (cm)
B

N. benthamiana used for virus purification. Electron microscopy of purified BlCMV-I negatively stained with molybdate revealed the presence of tubular inclusions with only trace amounts of host components (Fig. 11). At high magnification, striations of protein subunits were observed on individual tubes (Fig. 11-D). These regularly spaced striations were estimated to have a periodicity of approximately 5 nm. Striations with similar periodicity have been observed in cytoplasmic inclusions induced by several other potyviruses (Edwardson et al., 1968; Hiebert and McDonald, 1973; and Morales and Zettler, 1977). Few virus particles were observed in the purified preparations of BlCMV-I which were not reactive to BlCMV-As (Fig. 12). Purified preparations of BlCMV-I with the highest degree of purity were obtained from N. benthamiana, with yields of 5 to 20 A_{280} units were usually obtained from 100 g of fresh weight of N. benthamiana or V. unguiculata tissues. The ultraviolet absorption spectrum obtained for SDS disassociated BlCMV-I was typical of proteins, with a maximum at 277 nm and a minimum at 246 - 248 nm (Fig. 5). Polyacrylamide gel electrophoresis of SDS-disrupted inclusion proteins revealed a single subunit component estimated to have a MW of 70,000 daltons (Fig. 10).

Virus Particle Size and Stability in Sap

Electron microscopic examinations of purified preparations of BlCMV negatively stained with PTA indicated that 73% of 190 virus particles measured were between 700 and 800 nm with a modal length of 753 nm. Particle measurements of several leaf-dip preparations negatively stained with PTA and of grids prepared for SSEM with infected cowpea leaf tissue gave modal lengths of 758 and 780 nm,

Figure 11 - Electron micrographs of purified preparations of BlCMV cytoplasmic inclusions stained with molybdate. All purified preparations consisted of tubes, most of which were fragmented during the purification process. Note striations (St) on high magnification (D).

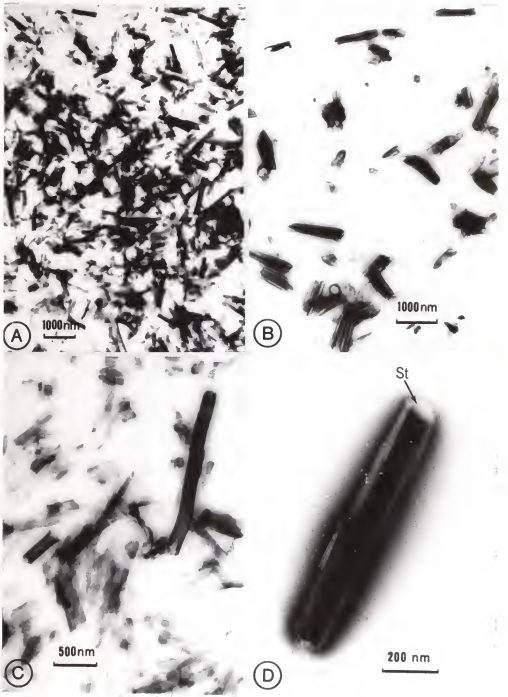
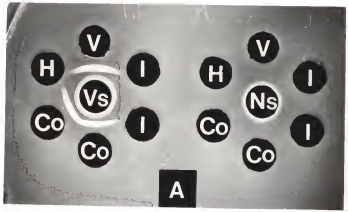

A
1000nm
B
1000nm
C
500nm
St
D
200 nm

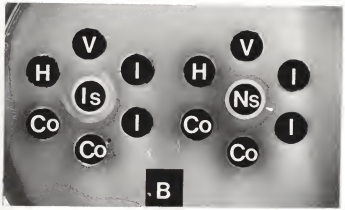
Figure 12 - Double immunodiffusion tests in agar medium containing 0.8% Noble agar, 1.0% NaN_3, and 0.5% SDS.

A - B) Serological tests to demonstrate specificity of BlCMV antiserum (Vs), and antiserum obtained against BlCMV induced cytoplasmic inclusions (Is). The peripheral wells were filled with the following antigenic solutions prepared in 1.5% SDS: (V) purified BlCMV, (I) purified preparations of BlCMV inclusions, (Co) extracts of BlCMV-infected cowpea, and (H) crude extract of healthy cowpea. All antigens were also tested against normal serum (Ns).

C) Serological relationship between BlCMV and a New York isolate of BlCMV. The center wells were filled with: BlCMV antiserum (Bls), and normal serum (Ns). The peripheral wells were charged with SDS-treated extracts from: (Bl) BlCMV-infected cowpea, (NY) isolate of BlCMV from New York in cowpea, and (H) healthy cowpea.

D) Serological relationship between a strain of bean common mosaic virus isolated from siratro (BCMV-S) and the BlCMV isolate from New York. The center wells were charged with: (Ss) BCMV-S antiserum, and (Ns) normal serum. The peripheral wells were charged with SDS-treated extracts from: (S) bean leaves infected with BCMV-S, (NY and N) cowpea leaves infected with the New York isolate of BlCMV, (H-lower) healthy bean, and (H-upper) healthy cowpea.


V
H
I
Vs
Co
I
Co
V
H
I
Ns
Co
I
Co
A


V
H
I
Is
Co
I
Co
V
H
I
Ns
Co
I
Co
B


Bl
H
Bl
Blis
H
NY
NY
H
Bl
Bl
Ns
H
NY
NY
C


S
H
S
Ss
H
NY
NY
H
S
S
Ns
H
NY
N
D

respectively, with 90% of the particle lengths ranging from 700 to 800 nm (Figs. 7 and 8). Some variation was observed with the particle size of purified virus stained with PTA and virus particles in leaf extracts prepared by SSEM and stained with uranyl acetate (Figs. 7 and 8). On the other hand, grids with less plant debris and higher concentrations of virus particles were obtained with SSEM than with the conventional leaf-dip preparation (Fig. 6). Using normal leaf-dip preparations at least four grids were prepared and 10 electron micrographs were taken to measure a maximum of 25 virus particles. On the other hand, 132 virus particles were measured by examining two micrographs obtained by SSEM.

In cowpea leaf extracts, BICMV had a TIP of 65 C, LIV of 48 hr, and DEP of 10^{-4}. Blackeye cowpea mosaic virus was still infectious after 10 min at 60 C but not at 65 C and lost its infectivity after 48 hr at room temperature, but not at 24 hr. Sap of cowpea leaves systemically infected with BICMV lost infectivity when diluted more than 10^{-3} with distilled water.

Serology

Antisera specific for BICMV were obtained against untreated virions and pyrrolidine degraded viral protein. Both antisera reacted with SDS- or pyrrolidine-treated BICMV in purified preparations or in plant sap in double and single radial diffusion tests (Figs. 12, 13, 14). Most bleedings were specific for viral antigens; however, some bleedings also reacted with extracts from healthy plants, suggesting the presence of antibodies specific for normal plant components. To remove these antibodies the antiserum was absorbed with plant

Figure 13 - Single radial diffusion tests in agar media containing different concentrations of SDS and antisera for black-eye cowpea mosaic virus (BlCMV-As) and cowpea mosaic virus (CPMV-As).

The media in (A, B, C) contain 0.8% Noble agar, 1.0% NaN_3, 0.3% SDS, and 10% BlCMV-As (A), 15% BlCMV-As (B), and 20% BlCMV-As (C). The media in (D, E, F) contain 0.8% Noble agar, 1.0% NaN_3, 0.5% SDS, and 10% BlCMV-As (D), 15% BlCMV-As (E), and 20% BlCMV-As (F). The wells in (A, B, C, D, E, F) were charged with: (1) extracts from BlCMV-infected cowpea prepared in 1.5% SDS 1/2 (w/v), (2) solution used in "1" diluted 1/2 with 1.5% SDS, (4) solution used in "1" diluted 1/4 with 1.5% SDS, (8) solution used in "1" diluted 1/8 with 1.5% SDS, and (H) extract from healthy cowpea prepared in 1.5% SDS.

The media in (G, H) contain 0.8% Noble agar, 1.0% NaN_3, 0.5% SDS, and 15% BlCMV-As + 15% CPMV-As (G), and 10% BlCMV-As + 10% CPMV-As (H). The media in (I, J) contain 0.8% Noble agar, 1.0% NaN_3, 0.3% SDS, and 10% BlCMV-As + 10% CPMV-As (I), and 8% BlCMV-As + 8% CPMV-As (J). The wells in (G, H, I, J) were charged with SDS-treated extracts from: BlCMV-infected cowpea (row no. 1), CPMV-infected cowpea (row no. 2), cowpea leaf tissue containing both BlCMV and CPMV (row no. 3), and healthy cowpea (row no. 4).

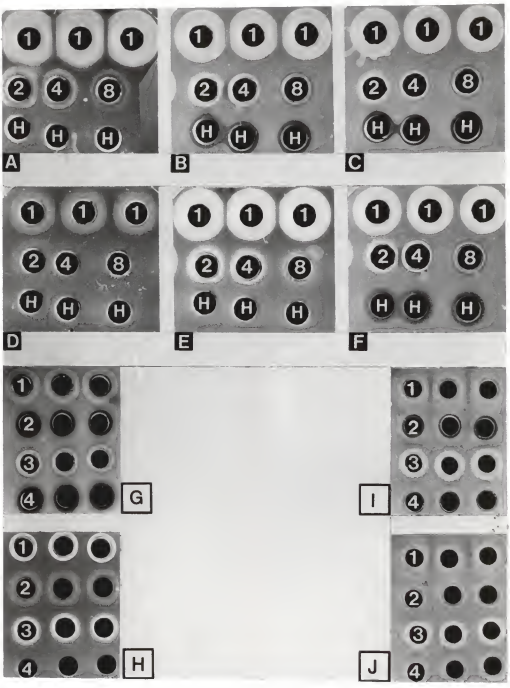
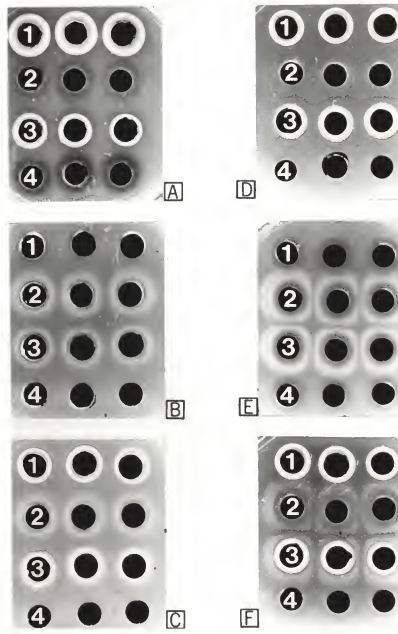

1 1 1
2 4 8
H H H
A
B
C
D
E
F
1
2
3
4
G
H
I
J

Figure 14 - Single radial diffusion tests with SDS and pyrrolidine degraded capsid protein of blackeye cowpea mosaic virus (BlCMV) and cowpea mosaic virus (CPMV).

The media in (A, B, C) contain 0.8% Noble agar, 1.0% NaN_3, 0.5% SDS, and 15% BlCMV-As (A), 15% CPMV-As (B), and 10% BlCMV-As + 10% CPMV-As (C). The wells were charged with SDS-treated extracts from: BlCMV-infected cowpea (row 1), CPMV-infected cowpea (row 2), BlCMV and CPMV in cowpea (row 3), and healthy cowpea (row 4).

The media in (D, E, F) contain 0.8% Noble agar, 0.2% NaN_3, 0.85% NaCl, and 15% BlCMV-As (D), 15% CPMV-As (E), and 10% BlCMV-As + 10% CPMV-As (F) prepared in 0.05 M Tris-HCl buffer, pH 7.2. The wells were charged with pyrrolidine-treated extract from: BlCMV-infected cowpea (row 1), CPMV-infected cowpea (row 2), BlCMV and CPMV in cowpea (row 3), and healthy cowpea leaves (row 4).


1
2
3
4
A
D
B
E
C
F

components purified from V. unguiculata by high speed centrifugation according to the method used by Purcifull et al. (1973). The high specificity of most of the antisera obtained against purified BlCMV preparations confirmed the efficiency of the methods used for its purification. The titers of antiserum varied depending on the bleeding date and on the rabbits, but 32 was the highest antiserum titer estimated by SDS-gel double immunodiffusion tests with a series of dilutions (1/2, 1/4, 1/8, 1/16, and 1/32) of BlCMV-infected cowpea tissue prepared in 1.5% SDS.

Antiserum specific for cytoplasmic inclusions induced by BlCMV was obtained from a rabbit injected with preparations of BlCMV-I purified from infected tissue of N. benthamiana. The BlCMV-I antiserum reacted specifically with purified preparations of BlCMV-I and crude sap of BlCMV-infected cowpea, but not with either purified preparations of BlCMV or crude sap of noninoculated plants (Fig. 12-B). The positive reactions with BlCMV-I were more evident after 48 hr of incubation. The results obtained with BlCMV antiserum also indicated that BlCMV was not serologically related to its cytoplasmic inclusions (Fig. 12-A). Attempts to obtain specific antiserum by injecting rabbits with BlCMV-I purified from infected cowpea tissue were unsuccessful. All three rabbits injected with BlCMV-I purified from infected cowpea developed high titers of antibodies specific for normal plant tissue antigens.

Single radial immunodiffusion studies in SDS-agar medium impregnated with the virus antiserum indicated that appropriate SDS and antiserum concentrations need to be previously selected for highest sensitivity and to avoid spurious reactions. The best results were

observed when the antigens were prepared in 1.5% SDS and the medium used had 0.3% SDS and 10% antiserum (Fig. 13-A) or 0.5% SDS and 15% antiserum (Fig. 13-E). The same results were consistently observed with different batches of plates with the same medium compositions. Similar results were also observed with CPMV using antiserum obtained for SDS-treated virus. On the other hand, different results were observed in SDS medium containing a mixture of BlCMV and CPMV antisera. All media containing 0.3% SDS were cloudy with all combinations of BlCMV and CPMV antisera used (Fig. 13-I, -J), indicating some type of interaction between SDS and antiserum proteins. However, even with the cloudy appearance, some virus-specific reactions were still observed (Fig. 13-I, -J). Clearer media were obtained with 0.5% SDS and 10 or 15% of each antiserum. The best reactions, however, were observed when both BlCMV and CPMV antisera were used at concentrations of 10% in media containing 0.5% SDS (Fig. 13-H). Strong precipitin rings were observed around the wells containing BlCMV or a combination of BlCMV and CPMV whereas weaker reactions were observed around the wells containing only CPMV (Figs. 13-H, 14-C). Unexpectedly, no reactions were observed around the wells containing only CPMV in a medium containing 15% of each antiserum and 0.5% of SDS (Fig. 13-G).

Virus-specific reactions using BlCMV and CPMV antisera were also obtained with the single radial diffusion method described by Shepard (1972). Precipitin rings around the virus-wells were observed when the antigens were prepared in 1.5 or 2.5% pyrrolidine and the medium used contained 0.8% Noble agar, 0.2% NaN_3, and 10 to 15% virus antisera prepared in 0.05 M Tris-HCl buffer, pH 7.2, containing 0.85% NaCl. Sharp, white precipitin rings were formed close to edges of

the wells containing BlCMV in agar medium prepared with BlCMV antiserum, whereas whitish halos with greater diameters were formed around the wells containing CPMV in agar medium impregnated with CPMV antiserum (Fig. 14-D, -E, -F). The same distinction between these two types of precipitin rings was observed when both antisera were added into the same medium, so that two concentric rings were formed around the wells containing both viruses (Fig. 14-F). The inner ring was the result of BlCMV-antibody specific reactions and the larger halos resulted from CPMV-specific reactions. This difference in types of precipitin rings could be related to the concentration of the antigens placed in the wells and to the reciprocal of antibody concentration (Shepard, 1972). Stronger and more compact rings were observed with CPMV when the antigens were diluted or the antiserum concentration was increased.

Reciprocal double immunodiffusion tests with SDS-treated antigens showed that BlCMV is serologically related to, but distinct from, the following potyviruses: BCMV-BV-1, BCMV-S, BYMV, DMV, LMV, PVY, SoyMV, TEV, and WMV-2 (Fig. 15). No reactions were detected, however, with certain potyviruses, including BiMV, PeMV, TuMV, WMV-1, CoMV, PVMV, and PWMV. Antiserum for BlCMV also reacted specifically with CAMV forming a distinct spur which extended past the heterologous reaction (Fig. 16-A). In all positive serological relationships observed in the reciprocal serological tests, spurs were formed in both directions (Fig. 15).

The serological distinctions observed between BlCMV and BCMV-S, and CAMV by spur formation were demonstrated by the intragel cross absorption technique (Fig. 16-B, -D). The heterologous antigens,

Figure 15 - Reciprocal double immunodiffusion tests with BlCMV and other potyviruses in medium containing 0.8% Noble agar 1.0% NaN_3, and 0.5% SDS prepared in 0.05 M Tris-HCl buffer, pH 7.2. All the antigenic solutions were prepared in 1.5% SDS. The center wells were charged with: (1) BlCMV antiserum, (2) PVY antiserum, (3) TEV antiserum, (4) WMV-2 antiserum, (5) DMV antiserum, (6) BCMV-BV-1 antiserum, (7) BCMV-S antiserum, (8) SoyMV antiserum, (9) BYMV antiserum, (10) BiMV antiserum, and (11) LMV antiserum. The top rows of wells in all cases were charged with SDS-treated extracts from: (a) BlCMV-infected cowpea, and (c) healthy cowpea. The bottom rows of wells were charged with SDS-treated extracts from: A) PVY-infected tobacco (b), and healthy tobacco (d); B) TEV-infected tobacco (b), and healthy tobacco (d); C) WMV-2-infected pumpkin (b), and healthy pumpkin (d); D) DMV-infected dasheen (b), and healthy dasheen (d); E) BCMV-BV-1 infected bean (b), and healthy bean (d); F) BCMV-S-infected bean (b), and healthy bean (d); G) SoyMV-infected N. benthamiana (b), and healthy N. benthamiana (d); H) BYMV-infected pea (b), and healthy pea (d); I) BiMV-infected Nicotiana hybrid (b), and healthy Nicotiana hybrid (d); and J) LMV-infected pea (b), and healthy pea (d).

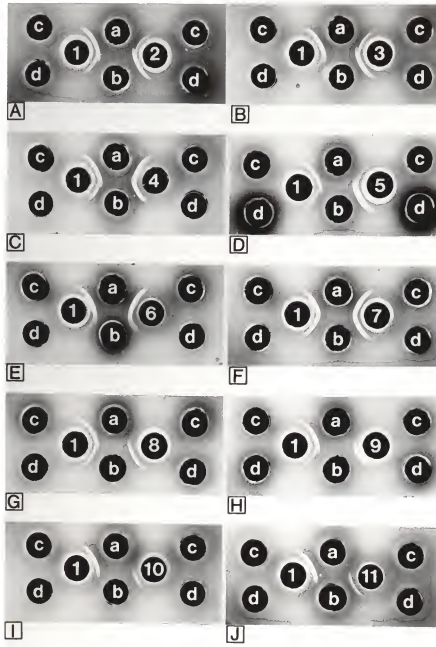

A
B
C
D
E
F
G
H
I
J

Figure 16 - Immunodiffusion tests with BlCMV, Moroccan isolate of CAMV and siratro strain of bean common mosaic virus (BCMV-S) in agar medium containing 0.8% Noble agar, 1.0% NaN_3, and 0.5% SDS prepared in 0.05 M Tris-HCl buffer, pH 7.2.

A) Serological tests with BlCMV and CAMV. The center wells were charged with: BlCMV antiserum (Bls), and normal serum (Ns). The peripheral wells were filled with SDS-treated extracts from: (Bl) BlCMV-infected cowpea, (Ca) CAMV-infected cowpea, and (H) healthy cowpea.

B) Intragel cross-absorption test with BlCMV and CAMV. The center wells were charged with: (1) BlCMV antiserum, (2) purified CAMV and 20 hr later BlCMV antiserum. The peripheral wells were filled with SDS-treated extracts from: (Bl) BlCMV-infected cowpea, (Ca) CAMV-infected cowpea, and (H) healthy cowpea.

C) Serological tests with BlCMV and BCMV-S. The center wells were charged with: (Bls) BlCMV antiserum, (Ss) BCMV-S antiserum. The peripheral wells were filled with SDS-treated extracts from: (Bl) BlCMV-infected cowpea, (S) BCMV-S-infected bean, (Hb) healthy bean, and (Hc) healthy cowpea. The arrows point to spurs.

D) Intragel cross-absorption test with BlCMV and BCMV-S. The center wells were filled with: (1) BlCMV antiserum, and (2) purified BCMV-S and 20 hr later BlCMV antiserum. The peripheral wells were charged with SDS-treated extracts from: (Bl) BlCMV-infected cowpea, (S) BCMV-S-infected bean, (Hb) healthy bean, and (Hc) healthy cowpea.

E) Serological tests with BCMV-S and CAMV using two different antisera for BCMV-S. The center wells were charged with: (1) BCMV-S antiserum from a rabbit inoculated with freshly purified preparations of BCMV-S, and (2) BCMV-S antiserum obtained from the same rabbit after a booster injection with purified BCMV-S stored at 4 C for 30 days. The peripheral wells were charged with SDS-treated extracts from: (S) BCMV-S-infected bean, (Ca) CAMV-infected cowpea, (Hc) healthy cowpea, and (Hb) healthy bean.

F) Serological test with fresh and stored purified preparations of BlCMV. The center well was charged with BlCMV antiserum, and the peripheral wells with SDS-treated new purified preparation of BlCMV (Np), old purified BlCMV (Op), and healthy cowpea extracts (H).


Ca
Ca
Ca
Ca
Bl
Bls
Ns
Ca
H
Bl
H
H
H
A
H
Bl
H
1
2
H
Ca
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B
S
S
S
Bl
Bls
Ss
S
Hb
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Hb
Hc
Hc
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Hc
Bl
Hc
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Hb
S
Hb
S
D
Hb
1
S
Hb
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Hc
Ca
Hc
E
H
Np
Bls
H
Op

which were placed in the center well prior to the antiserum, cross-reacted with and fully precipitated the cross-reacting antibodies at the region of optimal proportions close to the center well.

Serological distinction was also observed between a freshly purified preparation of BlCMV and purified BlCMV stored at 4 C for more than 30 days (Fig. 16-F). This suggested some enzymatic degradation of certain BlCMV antigenic determinants during the storage period.

Serological relationship studies between CAMV and BCMV-S using BCMV-S antisera obtained by different bleedings of the same rabbit indicated that the antiserum specificity varied according to the immunization program and the conditions of the antigenic solution used. A highly specific antiserum for BCMV-S was obtained from a rabbit injected with approximately 8 mg of freshly purified preparations of BCMV-S. Using this specific antiserum it was possible to show a complete serological distinction between BCMV-S and CAMV (Fig. 16-E). About three months after the initial immunization, a booster injection with a purified preparation of BCMV-S stored at 4 C for more than one month was given to the same rabbit. All antisera obtained 15 days or more after the booster injection reacted with CAMV, forming a spur between CAMV and BCMV-S when they were placed into adjacent antigen wells around the antiserum well (Fig. 16-E).

Light and Electron Microscopy

Light microscopic observations of epidermal leaf strip preparations from plants systemically infected with BlCMV revealed the presence of tubular cytoplasmic inclusions similar to those described by Edwardson et al. (1972) and Edwardson (1974) for BlCMV. Side views of groups of tubular inclusions were easily observed in BlCMV-infected

leaves (Fig.17), and at high magnification end views of them could be seen as small dots by changing the microscope focus. In ultrathin sections of BlCMV-infected tissue, these inclusions consisted of tubes attached to a central core, forming pinwheels (Fig. 18), similar to those induced by the potyviruses from Edwardson's subdivision-I (Edwardson, 1974). As reported by Edwardson (1974), the pinwheels contained arms with pronounced curvatures and tight scroll-like tubular inclusions. Only tubes were observed in purified preparations of cytoplasmic inclusions induced by BlCMV (Fig. 11).

Host Range and Resistant Cowpea Varieties

Blackeye cowpea mosaic virus was readily transmitted mechanically from cowpea 'Knuckle Purple Hull' to the following plants in which it was detected serologically and caused the following symptoms: Crotalaria spectabilis (mosaic); Glycine max (L.) Mer. (mild mottle and chlorotic spots); Macroptilium atropurpureum (DC.) Urb. (mosaic); Macroptilium bracteatum (L.) Urb. (mosaic); Nicotiana benthamiana (mottle); Ocimum basilicum (local lesions); Phaseolus vulgaris 'Black Turtle-2' (epinasty, necrosis, yellowing) and 'Bountiful' (chlorotic spots on inoculated leaves); Vigna unguiculata 'Black Local' (mosaic), 'Early Ramshorn' (mottle), 'Knuckle Purple Hull' (mosaic), and 12 Brazilian cowpea cultivars in which the reactions varied from symptomless to mosaic (Table 1). Small chlorotic lesions were found on the leaves of Chenopodium amaranticolor inoculated with purified preparations of BlCMV or cowpea sap containing BlCMV (Fig. 1-B).

Based on failure to induce symptoms and on negative serological results, BlCMV did not infect Arachis hypogaea L. 'Florunner', Capsicum

Figure 17 - Photomicrographs (A, B, C, D) of cytoplasmic inclusions in epidermal strips of cowpea leaves systemically infected with BlCMV, stained with a combination of calcomine orange and luxol brilliant green. A) cells with masses of cytoplasmic inclusions, B) details of mass of inclusions seen in "A", C) general view of the inclusion distribution in epidermal cells, and D) phase contrast micrograph of the area photographed in "A". (Ci) cytoplasmic inclusions, (CW) plant cell wall, (G) guard cells, and (Nu) nucleus.

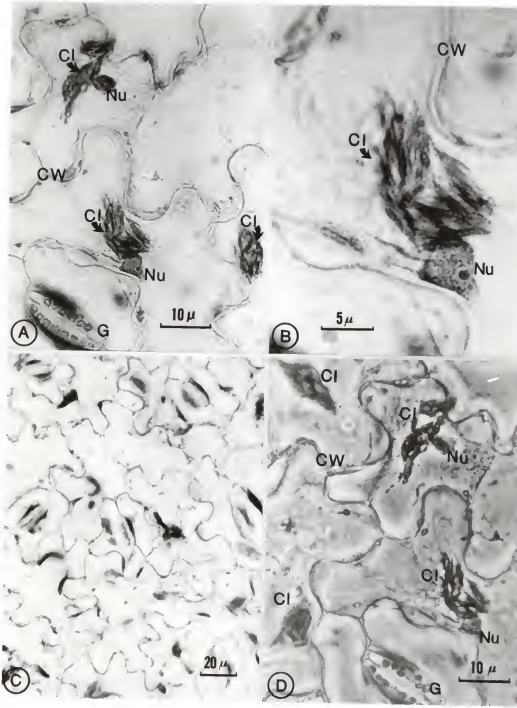

Cl
Nu
CW
Cl
Cl
Nu
G
10 μ
A
CW
Cl
Nu
5 μ
B
20 μ
C
Cl
Cl
CW
Nu
Cl
Cl
Nu
10 μ
G
D

Figure 18 - Electron micrographs of ultrathin sections of cowpea leaf cells infected with BICMV showing cross-sections (A, B, C) and longitudinal sections (D) of pinwheel inclusions.

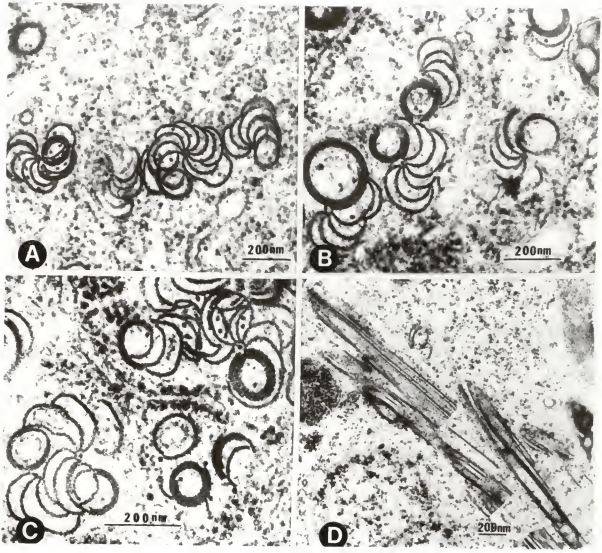

A
200nm
B
200nm
C
200nm
D
200nm

Table 1. Symptoms and results of serological assays on varieties of cowpea, Vigna unguiculata mechanically inoculated with BlCMV, BCMV-S, CAMV, and CPMV.

Cowpea Varieties	Symptoms(a)				Serology(b)			
	BlCMV	BCMV-S	CAMV	CPMV	BlCMV	BCMV-S	CAMV	CPMV
Black Local	Mt	-	M	-	+	-	+	-
Bola de Ouro	-	-	Mt	Mt	-	-	+	+
CE-73	-	Mt	Mt	Mt	+	+	+	+
CE-74	Mt	-	Mt	-	+	-	+	-
CE-175	M	-	Mt	Mt	+	-	+	+
CE-89	M	-	Mt	-	+	-	+	-
CE-3-53	Mt	-	M	-	+	-	+	-
Cream 40	Mt	-	M,Ld	M,Ld.De	+	-	+	+
Early Ramshorn	Mt	M	M	Ne,De	+	+	+	+
Crowder Pea	-	-	Mt	M,Ld	-	-	+	+
Ipeane VII	Mt	-	Mt	-	+	-	+	-
Jaguaribe	M	-	Mt	-	+	-	+	-
Knuckle Purple Hull	M	-	M,Ld	M,Ld,De	+	-	+	+
Pitiuba	Mt	-	M	-	+	-	+	-
Potomac	M	-	Mt	-	+	-	+	-
Serido	-	-	Mt	Mt	-	-	+	+
Snapper Long Pod	-	-	M,Ld	*	-	-	+	*
Sete Semanas	-	-	Mt	-	-	-	+	-
V-4 Alagoas	M	-	Mt	Chl	+	-	+	+
V-5 Parayba	M	-	M	Mt	+	-	+	+

(a) Chl= systemic chlorosis, De= plant death; Ld= leaf deformation, M= mosaic, Mt= mottle, Ne= systemic necrosis, and - = no symptoms.

(b) + = positive serological reaction in double diffusion tests,
- = no serological reaction in double diffusion tests.

* = not tested

annuum L. 'Early Calwonder'; Cucumis sativus L.; Cucurbita pepo L. 'Small Sugar'; Lupinus angustifolius L. 'Bitter Blue'; Lupinus luteus L. 'Sweet Yellow'; Phaseolus vulgaris 'Black Turtle-1', 'Green Northern 1140', 'Improved Tendergreen', 'Lake Shasta', 'Michelite 62', 'Pink Rosa', 'Pink Viva', 'Puregold Wax', 'Red Mexican U-34', 'Red Mexican U-35', 'Top-crop' and 'VC 1822'; Pisum sativum L. 'Alaska', 'Bonneville' and 'Ranger'; and Vicia faba L.

The reactions of cowpea varieties to mechanical inoculations of BlCMV, BCMV-S, CAMV, and CPMV are indicated in Table 1. All inoculated plants were assayed serologically for the presence of the viruses (Table 1).

Discussion

Blackeye cowpea mosaic virus and its cytoplasmic inclusions were successfully purified from systemically infected cowpea or N. benthamiana leaf tissues with the procedures outlined herein. The first method of virus purification (Fig. 2) gave good yields of highly purified BlCMV, and the combination of n butanol and chloroform-carbon tetrachloride (Fig. 4) was the better procedure for purification of BlCMV and its cytoplasmic inclusions from the same batch of tissue. The high degree of purity of the BlCMV preparations indicated by spectrophotometry, analytical centrifugation and PAGE analyses, as well as serological studies and electron microscopic observations, confirmed the efficiency of the purification procedures.

Aggregation of virus particles and virus and host components during purification appears to be a limiting factor for obtaining high

yields of viruses in the PVY group (Shepherd and Pound, 1960; van Oosten, 1972; Hiebert and McDonald, 1973; Uyeda et al., 1975; and Barnett and Alper, 1977). Hiebert and McDonald (1973) reported aggregation of virus particles after PEG precipitation. The losses of BlCMV by low speed centrifugation due to aggregation of virus particles were reduced by maintaining the virus in KPO_4, buffer, pH 8.2, after precipitation with PEG.

Another critical aspect on purification of potyviruses for obtaining maximum virus yield is the host used for virus increase. In order to obtain a good yield of BlCMV from the 'Knuckle Purple Hull' variety of cowpea the virus was inoculated into the source plants at the age of 3 to 4 days after emergence and the systemically infected leaf tissues were harvested 15 to 18 days after inoculation. Attempts to purify the virus from plants inoculated later than that or from tissue harvested more than 30 days after inoculation resulted in very poor yields of virus and cytoplasmic inclusions.

Electron microscopic examinations of purified preparations of BlCMV indicated a low percentage of virus fragmentation during the purification processes (Fig. 6-A). Particle measurements of purified BlCMV and of BlCMV particles on grids prepared for SSEM with infected cowpea leaf tissue gave two modal lengths (Figs. 7, 8) which differed by approximately 30 nm. Variations in lengths of virus particles have been extensively observed (Edwardson, 1974). As reviewed by Edwardson (1974), virus length variations may be attributed to several factors, including sample preparation, host influence, virus strain differences, and normal fluctuations in the electron microscope magnification. Increase of 50 to 100 nm in certain potyvirus particle lengths induced

by magnesium ions were reported by Govier and Woods (1971). They indicated that in the presence of Mg ions the particles were straight, contrasting with the flexuous particles observed in the absence of Mg ions. On antiserum-coated grids several antibodies combine with a single virus particle and, possibly, increase its length. Because of the specific antigen-antibody reaction the BlCMV particles were so strongly attached to the surface of the antiserum-coated grids that they could not be removed by repeated washing. On the other hand, positive staining of BlCMV particles with ethanolic uranyl acetate may have induced some changes in their lengths. Measurements of 25 BlCMV particles on grids prepared by conventional leaf-dip preparation with PTA gave a modal length similar to that estimated for purified BlCMV negatively stained with PTA. Milne and Luisoni (1977) emphasized that negative staining gives better preservation and better resolution of viral capsids than positive staining. Using SSEM with uranyl acetate as a negative stain, Milne and Luisoni (1977), observed no change in the normal lengths of TMV and a potexvirus. However, leaf-dip preparations often contain too few particles to photograph conveniently for virus particle measurements, whereas relatively large numbers of particles can be photographed by using the serologically specific electron microscopic technique. As indicated by Derrick and Brlansky (1976), the addition of sucrose in the extracting and washing buffers greatly reduced the amount of plant debris on the SSEM grids. High amounts of plant debris in electron microscopic preparations are frequent problems in establishing the dimensions of a virus.

Polyacrylamide gel electrophoresis of SDS dissociated cytoplasmic inclusions and viral coat proteins clearly indicated that the viral coat protein subunit was smaller than the inclusion subunit (Fig. 10). The PAGE results revealed that the inclusions were made of a single kind of protein with an estimated molecular weight of 70,000 daltons. Polyacrylamide gel electrophoresis of cytoplasmic inclusion preparations conducted by Hiebert and McDonald (1973) showed one protein component with molecular weight of 67,000 daltons for PVY; 67,000 for PeMV; 69,300 for BiMV; 69,600 for TEV; and 70,300 for TuMV. The PAGE studies also indicated that freshly purified BlCMV consisted of a main protein component with a molecular weight around 34,000 daltons. Two smaller protein components were also revealed by PAGE analysis of SDS denatured viral coat protein (Fig. 10). Since only traces of the faster moving proteins were observed with fresh purified BlCMV, and greater amounts of these proteinaceous components were revealed by PAGE analyses of stored purified BlCMV preparations (Fig. 10), it is assumed that the smaller components are due to the degradation of the slow moving protein during purification and storage. Hiebert and McDonald (1976) observed that some possible enzymatic degradation of TuMV capsid protein occurred during storage of purified virus preparations. The lower sedimentation coefficient estimated for stored purified BlCMV preparations (Fig. 9) is further evidence of proteolytic degradation of viral coat protein during storage at 4 C. According to Hiebert and McDonald (1976), it is likely that "s_{20} values reported for potyviruses that are near 140 S represent virus with partially degraded capsid protein, whereas those near 160 S represent virus with intact capsid protein." This proteolytic degradation also changes

the antigenic properties of viral coat protein (Hiebert and McDonald, 1976; and Purcifull and Batchelor, 1977). Using antiserum obtained for freshly purified BlCMV, serological distinction was observed between freshly purified preparations of BlCMV and purified BlCMV stored at 4 C for more than 30 days (Fig. 16-F). The serological distinctions between different antigen preparations of the same virus observed herein are of great significance for serological identification and characterization of potyviruses as pointed out by Hiebert and McDonald (1976) and Purcifull and Batchelor (1977). It is important to keep in mind that purification, storage of either purified virus preparations or crude sap containing virus, and mailing of virus-infected fresh plant tissues may all result in modifications in the antigenic properties of viral coat protein. To solve this problem, the preservation of plant virus antigens by lyophilization of crude extracts from infected plants (Purcifull et al., 1975) or purified virus preparations is recommended. Blackeye cowpea mosaic virus has been maintained in lyophilized condition either in crude sap or purified preparation over two years during the course of this study without any perceptive change in its antigenic properties.

Another factor that should be considered during serological relationship studies between viruses in the PVY group is the specificity of antisera. Variations in the degree of cross reactivity exhibited by different antisera obtained against the same virus have been attributed to differences between individual animals (van Regenmortel and von Wechmar, 1970), route and number of injections used in the immunization program (Hollings and Stone, 1965) and time of bleeding

(Tremaine and Wright, 1967; and Koenig and Givord, 1974). The results of the present study indicated that the immunization program and the conditions of the antigenic solution used for rabbit immunization may also affect the antiserum specificity. A highly specific antiserum for BCMV-S was obtained from a rabbit immunized with freshly purified BCMV-S, whereas antiserum with a broader cross-reactive spectrum was obtained from the same rabbit after a booster injection with a purified preparation of BCMV-S stored at 4 C for more than one month. The use of such antisera would make it difficult to distinguish between certain plant viruses in SDS immunodiffusion tests. The serological distinction between BlCMV and BCMV-S was impossible to detect in SDS double-immunodiffusion when the BCMV-S antiserum with a wider cross-reactivity was used. On the other hand, an antiserum with a wide spectrum of activity should be useful for identification of virus-infected tissue used for plant propagation and possibly for identification of virus at the group level. As any virus-infected plant organ is undesirable for plant propagation the specific virus identification may not be necessary in such cases. For example, the BCMV-S antiserum was successfully used to identify cowpea seeds infected with BlCMV.

Unilateral serological relationships observed between BlCMV and SoyMV (Fig. 15-G) and with BlCMV and BYMV (Fig. 15-H) showed the nenessity of reciprocal tests for demonstrating the absence of serological relationship between two viruses. According to Matthews (1970) "to demonstrate that two viruses are serologically unrelated, reactive antisera must be prepared against each of the viruses under test." Reciprocal tests are also important to show distinction between two closely related viruses. It was more difficult to observe a spur

between BlCMV and WMV-2 when both viruses were tested against antiserum to WMV-2 than when they were tested against BlCMV antiserum (Fig. 15-C). Similar results were observed with BCMV isolates and BlCMV (Fig. 15-E, -F) which may explain the identical reaction reported by Uyemoto et al. (1973).

It is noteworthy that BlCMV and BYMV are serologically distinct, though related. This supports the contention of Edwardson et al. (1972) and Zettler and Evans (1972) that BlCMV and BYMV should be considered distinct viruses.

Serological differences between closely related viruses are better detected with antisera of fairly low titer (Matthews, 1970). On the other hand, he also stated that a high titer antiserum is preferable for demonstrating distant serological relationship. This can be illustrated by the serological tests carried out with BlCMV and CAMV isolates using a BlCMV antiserum with a titer of 32. By diluting the antiserum to 1/4, no reaction was observed with the heterologous virus (CAMV) whereas a fairly good reaction was still detected with the homologous antigen. The absence of reaction between BlCMV and LMV-antiserum (Fig. 15-J) may be a result of the low titer of the antiserum.

The intragel cross-absorption test was effective for demonstrating distinctions between two closely related viruses (Fig. 16-B, -D). This is additional evidence that serological distinctions that are undetectable in conventional double-immunodiffusion tests may be clearly revealed by intragel cross-absorption. Using this test, Matthews (1970) revealed a serological difference between type TMV and a nitrous acid induced mutant which showed a reaction of identity when tested against

unabsorbed TMV antiserum. For a full precipitation of the crossreacting antibodies close to the center well, a fairly high concentration of the heterologous antigen should be used to fill the antiserum well. This is illustrated by the intragel cross-absorption tests with BlCMV antiserum shown in Figure 16. A precipitin ring was formed very close to the center well when a highly concentrated purified preparation of BCMV-S (0.5 - 1.0 mg/ml) was used to absorb BlCMV antiserum (Fig. 16-D) whereas the ring formed approximately 2 mm away from edge of the well when BlCMV antiserum was absorbed with a less concentrated preparation of CAMV (0.01 - 0.05 mg/ml) (Fig. 16-B). In both cases, though, the intragel cross-absorption test showed serological distinction between the viruses. The intensity of the reaction between the homologous antigen and the cross absorbed antiserum may give some information about the degree of relationship between the viruses. Weaker homologous reaction indicates closer serological relationship. Based on this, the results of the present study clearly indicate that BlCMV is more closely related serologically to BCMV-S than to CAMV (Fig. 16-B, -D). The different degrees of serological relationships are also indicated by the intensity of the precipitin lines spurring over the heterologous virus reactions in straight diffusion tests (Fig. 16-A, -C). Serological relationship between different potyviruses has been commonly observed (Bercks, 1960; Purcifull and Shepherd, 1964; Purcifull and Gooding, 1970; Uyemoto et al., 1972; and Shepard et al., 1974), and the cross - absorption of an antiserum with heterologous viruses has also been used to study serological relationship between plant viruses in tube precipitin tests (Wetter, 1967; and Alba and Oliveira, 1977), and in combination with gel diffusion tests (van Regenmortel, 1966; Wetter,

1967; Nelson and Knuhtsen, 1973; Shepard et al., 1974; and Jones and Diachun, 1977). The intragel cross-absorption technique was also observed to be useful in demonstrating cross-protection between serologically distinct strains of plant viruses (Lima and Nelson, 1975).

The fact that most of the antisera obtained against purified BlCMV preparations did not react with extracts of noninfected cowpea tissue can be added to confirm the efficiency of the virus purification procedures described herein. On the other hand, the high population of antibodies for normal plant antigens developed by the rabbits injected with BlCMV-I purified from infected cowpea was an indication that virus-infected cowpea tissue may have a high concentration of host antigens, which were difficult to separate from the BlCMV cytoplasmic inclusions. However, using N. benthamiana as a source plant for BlCMV-I purification, antiserum specific for BlCMV-I was obtained. This is an additional indication of the useful application of N. benthamiana in plant virus research. Nicotiana benthamiana has been artificially infected with more than 50 plant viruses (Quacquarelli and Avgelis, 1975; and Christie and Crawford, in press), showing its great potential for cytological, serological, and physiological studies of different viruses in the same biological system.

The foot-pad route of rabbit immunization (Ziemiecki and Wood, 1975) used to obtain the antiserum specific for BlCMV-I was an efficient procedure. The high yield of antibodies obtained for BCMV-S using the same route of immunization (Lima et al., unpublished) is additional evidence that a high titer antiserum can be obtained at the expense of very little antigen.

Reciprocal immunodiffusion tests with antisera specific for BlCMV and BlCMV-I (Fig. 12-A, -B) confirmed the findings of Hiebert et al. (1971), Purcifull et al. (1973), Batchelor (1974), and McDonald and Hiebert (1975) that the inclusion body proteins are immunochemically distinct from viral coat protein and host proteins.

The results of single radial immunodiffusion tests indicated that agar-media impregnated with mono-specific antiserum or with a mixture of antisera can be used for serodiagnosis of two morphologically distinct legume viruses. Single radial immunodiffusion tests were first used in plant virology by Shepard (1969) for serodiagnosis of potato virus X in potato tuber sprouts. Subsequently the same method was successfully used to identify plant tissue infected with carlaviruses (Shepard, 1970; and Shepard et al. 1971) potyviruses (Uyemoto et al., 1972; and Casper, 1974), a cucumovirus (Richter et al., 1975), a hordeivirus (Slack and Shepherd, 1975), and tobamovirus (Granett and Shalla, 1970; and Clifford, 1977). Radial-immunodiffusion plates containing a mixture of antisera to two or three filamentous viruses have been used for detection of potato viruses X, S, and M (Shepard, 1972). This, however, appears to be the first report of a multiple-antisera medium for detection of both an isometric and a rod-shaped plant viruses.

Shepard (1969) observed that single radial immunodiffusion was more sensitive than double immunodiffusion for detection of PVX in infected plant tissue, but Richter et al. (1975) obtained better results with double diffusion tests than with single diffusion for serological detection of CMV in naturally infected herbaceous plants. No attempts to compare these two serological techniques were made in the present study. Some observations, however, indicated that single radial

immunodiffusion requires fairly large amounts of antiserum and that the proper antiserum concentration needs to be previously determined for highest sensitivity and to avoid spurious reactions in SDS-agar media. Better results in multi-antisera media were obtained when pyrrolidine was used as denaturant of virus coat protein.

The three serologically related but distinct legume viruses, BlCMV, BCMV-S, and CAMV can also be differentiated by some biological properties. The CAMV isolate was well adapted to cowpea, infecting and causing symptoms in all 20 inoculated cowpea varieties. On the other hand, five cowpea varieties showed immunity to BlCMV, and only two were infected with BCMV-S, which caused very mild symptoms (Table 1). The different symptomatological reactions induced by CAMV and BlCMV in some of the varieties (Table 1) clearly indicate that they can be used to distinguish these two potyviruses. It was observed, however, that some of the symptoms induced by the viruses varied with temperature, light conditions, and age at which the plants were inoculated, but no variation was observed with the immunity of any cowpea variety. The cowpea varieties that showed immunity to BlCMV (Table 1) should be included in a cowpea breeding program or in a control program for this virus in the southeastern United States. Cowpea lines with resistance to other viruses have been selected in different parts of the world (Williams, 1977a; and Beier et al., 1977). Virus-resistant lines with resistance to other plant pathogens have also been identified (Williams, 1977a and 1977b).

Attempts to compare BlCMV with the East African type of CAMV were impossible because all samples of virus-infected leaf-tissue arrived in high degree of decomposition with the virus already inactivated.

Serological studies with such decomposed leaf tissue and BlCMV antiserum gave results similar to those obtained with the CAMV isolate (Fig. 16-A) obtained originally from Morocco. As the inactivation of the virus in the decomposed tissue may have destroyed some of its antigenic determinants, no conclusive results about its serological relationship with BlCMV can be derived from these tests.

Light and electron microscopy of cowpea and other host cells infected with any one of these three legume potyviruses revealed that their cytoplasmic inclusions are morphologically similar. In ultrathin sections, their inclusions consisted of pinwheels similar to those induced by the potyviruses from Edwardson's subdivision-I (Edwardson, 1974). The cytoplasmic inclusions induced either by BCMV-S or CAMV, however, failed to react with antiserum for BlCMV induced inclusions. The low titer of the inclusion antiserum, however, may be one of the reasons for the absence of reactions. Despite the great similarity in the ultrastructures of pinwheel inclusions induced by BlCMV and CAMV, they showed some difference at the light microscope level. Whereas BlCMV induced big masses of cytoplasmic inclusions in 'Knuckle Purple Hull' (Fig. 17), only scattered small bundles of inclusions were observed in the cells of this host infected with CAMV. This is also an indication of no direct correlation between the severity of the symptoms and abundance of cytoplasmic inclusions induced by these potyviruses, since 'Knuckle Purple Hull' is more susceptible to CAMV than to BlCMV (Table I). A similar phenomenon was observed with these two viruses in C. spectabilis. In addition to this, the nuclear inclusions readily observed in cells of C. spectabilis systemically

infected with BlCMV (Christie and Edwardson, 1977) were not seen in leaf tissue of this host infected with CAMV.

In summary, BlCMV is a potyvirus that belongs to Edwardson's subdivision-I (Edwardson, 1974) and has a modal length of approximately 750 nm. The BlCMV particles have a single sedimenting peak with s_{20} = 157 - 159 S and have a main protein component with a MW of 34,000 daltons. Its cytoplasmic inclusions are made of tubes which show striations with periodicities of approximately 5 nm and consist of a single type of protein estimated to have a MW of 70,000 daltons. The virus also induces nuclear inclusions in certain hosts including C. spectabilis. Blackeye cowpea mosaic virus is serologically unrelated to seven potyviruses and serologically related to, but distinct from eight other potyviruses in SDS-immunodiffusion. The virus has a narrow host range outside Leguminosae, is seed-borne in at least two cowpea varieties and is transmitted by aphids in a nonpersistent manner. Based on its physical, biological, cytological and immunochemical properties, BlCMV can be differentiated from any other virus that infects cowpea. The antisera prepared for BlCMV and its cytoplasmic inclusions were essential tools for the development of the serological techniques for detection of virus-infected seeds described in Chapter II.

CHAPTER II

IMMUNOCHEMICAL AND CYTOLOGICAL TECHNIQUES FOR DETECTION OF LEGUME VIRUSES IN INFECTED SEEDS

Introduction

The transmission of plant viruses through seed of infected host plants was first demonstrated by Reddick and Stewart (1919), who showed that bean common mosaic virus (BCMV) was transmitted by approximately 50% of seeds from infected Phaseolus vulgaris. Since then, the phenomenon of seed transmission of plant viruses has received considerable attention and an appreciable number of viruses have been demonstrated to be seed-borne to some extent (Fulton, 1964; Bennett, 1966; Shepherd, 1972; and Phatak, 1974). Virus can be introduced into a crop at an early stage of plant development through infected seeds. Thus, the production of virus-free seeds, or seed lots with very low virus content may provide a very effective control of seed-borne plant viruses. Seed certification programs have been developed to test seed lots for the presence of viruses and to select virus-free seeds. Barley stripe mosaic virus, which is responsible for a serious disease in Montana (Afanasiev, 1956), and lettuce mosaic virus(LMV), the causal agent of an important disease of lettuce (Grogan et al., 1952), are good examples of virus diseases against which seed certification programs have been successful (Zink et al., 1956; Hamilton, 1965; Phatak, 1974; and Slack and Shepherd, 1975).

Barley stripe mosaic virus has no known vector, such as insects or mites, but it has a high rate of seed transmission, which severely reduces crop production. On the other hand, LMV is transmitted by a low percentage of seeds, but is regularly spread further in the field by aphids, resulting in substantial losses (Zink et al., 1956).

Several methods (Phatak, 1974) have been developed to detect the presence of infected seeds to control plant diseases caused by seed-borne viruses: Growing-on tests. Seeds are planted in greenhouses or under other insect-proof conditions and the first leaves of the seedlings are observed for the characteristic symptoms which vary according to the host-virus combination. This method can fail under environmental conditions that adversely affect the symptom development and with latent strains of a virus that do not produce visible symptoms. Indicator-inoculation tests. Seeds are ground up with buffer solution and mechanically inoculated in the indicator hosts. Although this method has been used extensively for LMV (Phatak, 1974), it is very time consuming and requires considerable greenhouse space. Serological tests. Immunochemical tests have also been developed for detecting virus in extracts from single seeds (Scott, 1961, and Lister, 1977), and individual seed embryos (Hamilton, 1965). However, no successful results have been obtained in double immunodiffusion systems with long flexuous rod viruses such as those from the potyvirus group (Phatak, 1974). Electron microscopy. A serologically specific electron microscopic technique developed by Derrick and Brlansky (1976) has been successfully used to detect the presence of virus particles in extracts of groups of seeds (Brlansky and Derrick, 1976).

The main purpose of the present investigation was to develop efficient and rapid serodiagnostic techniques to assay legume seed lots for the presence of virus-infected seeds. Seeds of cowpea, V. unguiculata 'Knuckle Purple Hull' infected with BlCMV were used as a model host-virus combination. Immunochemical techniques for detection of BlCMV, BCMV, and SoyMV in hypocotyls of germinated virus-infected seeds of V. unguiculata, P. vulgaris, and G. Max, respectively, are described in this chapter. Abstracts of portions of this research have already been published (Lima and Purcifull, 1977a, 1977b).

Literature Review

The phenomenon of seed transmission of plant viruses was first demonstrated by Reddick and Stewart (1919), who presented strong evidence of seed transmission of BCMV in Phaseolus vulgaris. Since then, a large body of information has been accumulated about the transmission of numerous plant viruses through the seeds of infected host plants (Fulton, 1964; Bennett, 1966; Shepherd, 1972; Baker, 1972; and Phatak, 1974). Among the 183 plant viruses described in the Commonwealth Mycological Institute up to September, 1977, (Doi et al., 1977), 51 viruses have been experimentally demonstrated to be seed-borne to some extent. Several plant viruses are known to be seed-borne in many leguminous host plants, but this review will cover only those viruses transmitted through seeds of Vigna spp., Glycine max, and Phaseolus vulgaris.

Seed-Borne Viruses in Vigna spp.

Gardner (1927) apparently was the first to report the transmission of a cowpea virus through seeds of cowpea. Since then, many viruses which naturally infect cowpea have been demonstrated to be seed-borne in this host.

A virus isolated from cowpea in Trinidad was demonstrated to be transmitted through 8% of seeds of asparagus-bean (Vigna sesquipedalis) obtained from virus-infected plants (Dale, 1949). The virus is believed to be a representative strain of cowpea mosaic virus (Agrawal, 1964; and van Kammen, 1971, 1972). It seems that the seed transmissibility of CPMV is erratic and depends on the type of virus isolate and the cowpea variety involved. A cowpea mosaic virus isolated from cowpea grown in Arkansas was seed-borne in this host (Shepherd, 1964). Approximately 620 'Blackeye' cowpea plants grown from seeds harvested from plants artificially inoculated with CPMV failed to develop mosaic symptoms (Perez and Cortes-Monllor, 1970). On the other hand, Haque and Persad (1975) observed that the rate of seed transmission of CPMV varied from zero to 5.8% depending on the cowpea varieties and selections.

Anderson (1957) reported the seed transmission of three cowpea viruses, including a strain of CMV, which was transmitted through 4 - 28% of cowpea seeds from artificially infected plants. A virus closely resembling a strain of CMV was seed-borne in cowpea with a transmission rate of 5 to 16% (Chenulu et al., 1968). On the basis of symptoms observed on cowpea plants grown from commercial seeds, Gay and Winstead (1970) reported seed transmission of CMV, a cowpea

strain of southern bean mosaic virus and a virus referred to as a cowpea strain of bean yellow mosaic virus. A strain of CMV isolated from cowpeas in Morocco was transmitted through approximately 25% of the seeds harvested from artificially inoculated cowpeas (Fischer and Lockhart, 1976b). The seed transmissibility of the cowpea strain of SBMV was first demonstrated by Shepherd and Fulton (1962).

McLean (1941) observed that a cowpea virus was transmitted through approximately 5% of seeds of highly susceptible cowpea varieties, but slightly susceptible or somewhat resistant varieties produced lower percentages of virus-infected seeds. Snyder (1942) observed only 3 to 4% transmission of a cowpea virus through commercial seeds and 37 to 41% transmission of the same virus through seeds obtained from virus-infected plants. Yu (1946), however, found no difference in the percentages of virus-infected cowpea plants grown from seeds obtained from artificially or naturally infected plants. These three viruses were also transmitted by aphids (McLean, 1941; Snyder, 1942; and Yu, 1946). Similar aphid-transmitted viruses reported from India were also demonstrated to be seed-borne (Nariani and Kandaswamy, 1961; and Verma, 1971). A rod-shaped virus isolated from cowpea in northern Italy was designated cowpea aphid-borne mosaic virus (CAMV) and studied by Lovisolo and Conti (1966) who demonstrated its seed transmissibility in cowpea. The transmissibility of CAMV through seeds of cowpea has been confirmed by several studies involving different strains of the virus and varieties of the host (Kaiser et al., 1968; Tsuchizaki et al., 1970; Bock, 1973; Bock and Conti, 1974; Phatak, 1974; Khatri and Singh, 1974; and Fischer and Lockhart, 1976a).

The most important cowpea virus in the southeastern United States was first isolated in Florida by Anderson (1955a) who later designated it blackeye cowpea mosaic virus (BlCMV) (Anderson, 1955b) and demonstrated its seed transmissibility in cowpea (Anderson, 1957). The seed transmissibility of BlCMV was confirmed by subsequent studies at the University of Florida, Gainesville. Zettler and Evans (1972) found as much as 18% of BlCMV-infected seeds in lots of certified cowpea seeds, and Uyemoto et al. (1973) reported 28% of seed transmission for this virus in cowpea 'Knuckle Purple Hull'.

Cowpea mild mottle virus, a carlavirus isolated from cowpea in Ghana, was demonstrated to be transmitted through seeds of cowpea, bean, and soybean in variable but sometimes large proportions (Brunt and Kenten, 1973, 1974). Cowpea banding mosaic virus, which was identified as a member of the cucumovirus group in India, was transmitted through high percentages of seeds from infected cowpea varieties (Sharma and Varma, 1975). A new isometric cowpea virus isolated from cowpea seeds from Iran and designated cowpea ringspot virus had a seed transmission rate of 15 to 20% in three cowpea cultivars (Phatak, 1974; and Phatak et al., 1976).

Thus, among the four filamentous and the six isometric viruses known to naturally infect cowpea, only TMV (filamentous) and cowpea chlorotic mottle virus (isometric) have not been demonstrated to be seed-borne in cowpea. Kuhn (1964b) found no evidence of seed transmission of cowpea chlorotic mottle virus (CCMV) in more than 2,100 seeds harvested from infected cowpea, and Gay (1969) observed no virus symptoms in 3,000 cowpea plants grown from seeds harvested

from CCMV-infected plants. According to Gay (1969), CCMV was not transmitted through seeds of cowpea because of virus inactivation during seed maturation.

Seed-Borne Viruses in Glycine max.

Soybean mosaic virus (SoyMV) which is probably common wherever soybean is cultivated (Bos, 1972) was first demonstrated to be seed-borne in soybean by Gardner and Kendrick (1921). Subsequently, they observed that the virus could overwinter in the virus-infected seeds lying in the field (Kendrick and Gardner, 1924). Although Kendrick and Gardner (1924) had also observed that mottled seeds were produced by both healthy and virus-infected plants and that diseased seedlings were produced by mottled as well as clean seeds, the association of seedcoat mottling and virus infection has been reported (Koshimizu and Iizuka, 1957; Ross, 1963, 1968, 1969; and Kennedy and Cooper, 1967). A mechanical selection of nonmottled soybean seeds has been suggested as a measure for controlling SoyMV in Brazil (Lima-Neto and Costa, 1976). However, Ross (1970) observed that SoyMV was equally transmitted through mottled and nonmottled seeds from virus-infected soybean plants, and concluded that the percentage of seed transmission for SoyMV in soybean could not be estimated from the amount of mottled seeds. Working with a Brazilian isolate of SoyMV, Porto and Hagedorn (1975) reported the production of mottled seeds by supposedly non-infected soybean cultivars and observed that SoyMV was not transmitted through seeds of virus-infected soybean cv, 'Bienville', which produced seeds with a high percentage of mottling. Bean pod mottle virus,

which is not seed-borne in soybean, also increased the percentage of mottled seeds (Ross, 1963).

Ghanekar and Schwenk (1974) showed a rate of seed-transmission for tobacco streak virus varying from 2.6 to 30.6% according to the soybean cultivar.

Among the nine viruses known to infect and cause diseases in soybean in Japan, SoyMV, soybean stunt virus, soybean mild mosaic virus, peanut stunt virus, southern bean mosaic virus, and a strain of alfalfa mosaic virus were reported to be transmitted to some extent through seeds of soybean varieties (Koshimizu and Iizuka, 1963; Iizuka and Yunoki, 1974; Takahashi et al., 1974; Iizuka, 1974; and Tamada, 1977).

The transmission of nepoviruses through seeds of their host is well documented. Tobacco ringspot virus (TRSV), a nepovirus (Fenner, 1976) responsible for a disease commonly called bud blight of soybean, was first reported to be transmitted through seeds of artificially inoculated soybean plants in 1954 (Desjardins et al., 1954). These results were confirmed by subsequent studies (Kahn, 1956; Owusu et al., 1968; Athow and Bancroft, 1959; and Demski and Harris, 1974). Kahn (1956) also demonstrated the seed-transmissibility of tomato ringspot virus in soybean and observed that soybean seeds from plants infected with either tobacco or tomato ringspot virus had a lower percentage of emergence than seeds from virus-free plants. Three other nepoviruses: arabis mosaic, raspberry ringspot and tomato black ring viruses were experimentally demonstrated to be transmitted through seeds of soybean plants artificially inoculated with these viruses (Lister, 1960; and Lister and Murant, 1967).

Seed-Borne Viruses in Phaseolus vulgaris

A large body of evidence (Bos, 1971) has confirmed the first demonstration of seed transmission of BCMV in P. vulgaris by Reddick and Stewart (1919). Burkholder and Muller (1926) reported that seeds from bean plants showing mosaic gave rise to 50% diseased plants. The virus remained viable within bean seeds for a period of at least 30 years (Pierce and Hungerford, 1929). Fajardo (1930) observed that both infected and noninfected bean seeds germinated with equal readiness and vigor, and concluded that the viability of the seeds was not directly affected by the presence of BCMV. Fajardo (1930) and others (Crowley, 1957, 1959; and Schippers, 1963) found that plants infected at early vegetative growth produced more virus-infected seeds than those infected at later stages.

It has been demonstrated that BCMV is transmitted irregularly through seeds harvested from virus-infected plants. Fajardo (1930) observed that some of the seeds in a single pod from bean plants infected with BCMV were virus-infected and some were noninfected. By crossing virus-infected and healthy bean varieties, Nelson and Down (1933) provided evidence that BCMV was equally transmitted by ovule or pollen from infected parent plants. Similarly, Medina and Grogan (1961) obtained high percentage of seed transmission of BCMV through either the pollens or ovules of infected plants, but also observed that the pollens usually transmitted the virus to a largernumber of progeny plants than the ovules. Cross-pollination experiments performed by Schippers (1963) revealed that the embryo infection with BCMV and consequently the seed-transmission might originate from

an infected egg-cell or an infected pollen grain. Studies to determine the distribution of BCMV in developing reproductive tissues suggested that successful seed transmission was insured by the presence of infective virus within the embryo itself (Ekpo and Saettler, 1974).

A correlation between symptom severity and percentage of seed transmission of BCMV has been observed in different bean varieties (Smith and Hewitt, 1938). Medina and Grogan (1961) suggested that the percentage of seed transmission of BCMV was greatly affected by the differences in varietal susceptibility of the beans. However, Zettler (1966) found similar percentages of seed transmission of BCMV in 5 different bean varieties.

Using the bean cultivar VC1822 as an indicator host for BCMV, Provvidenti and Cobb (1975) demonstrated a rate of seed transmission from 7 to 22% for BCMV in tepary bean (Phaseolus acutifolius Gray var. latifolius Freem.), and observed that the virus was carried in the embryo but not in the testa. Two BCMV isolates obtained from bean grown in the Netherlands were observed to be transmitted through approximately 20 to 80% of seeds harvested from infected bean plants (Drijfhout and Bos, 1977).

A strain of CMV isolated from bean plants grown in Spain was also demonstrated to be seed-borne in one of twelve bean varieties studied by Bos and Maat (1974). As they tested only a few seeds from virus-infected plants of each bean variety, they suggested that a low percentage of seed transmission could possibly occur in other varieties.

The transmissibility of SBMV through seeds of bean was first demonstrated by Zaumeyer and Harter (1943). Cheo (1955) found a very

high content of SBMV in the embryos of immature seeds from virus-infected bean plants, but observed that the virus concentration dropped to a low level or disappeared as the seed matured and dehydrated. Cheo's finding was confirmed by Crowley (1959), who found that approximately 100% embryo infection occurred when bean plants were inoculated with SBMV at any time prior to flowering, but no seed transmission of this virus occurred when samples of mature dry seeds were sown. These findings were supported by the results of McDonald and Hamilton (1972), which confirmed that in mature bean seeds, infectious SBMV is confined to the seed coat.

An ilarvirus was also reported to be seed-borne in bean. Tobacco streak virus, the causal agent of a severe disease outbreak of pinto bean in State of Colorado, U.S.A., in 1947, was demonstrated to be transmitted by approximately 26% of seeds from plants grown from virus-infected seeds (Thomas and Graham, 1951).

In summary, seed-transmission is an important factor in the perpetuation and dissemination of numerous viruses that cause diseases in cowpea, soybean, and bean. The following methods for detecting selected seedborne viruses in legumes may be useful in virus disease control programs and as research tools.

Materials and Methods

Source of Seed and Seed Germination

Seeds of cowpea, *V. unguiculata* 'Knuckle Purple Hull' and 'Early Ramshorn', harvested from BlCMV-infected plants were used in the present tests. All seed lots showing BlCMV-transmission were obtained either

in fields of 'Knuckle Purple Hull' cowpeas in Gainesville, Florida, or from Gilvan Pio Ribeiro, University of Georgia, Athens. As controls, virus-free 'Knuckle Purple Hull' seeds produced in Texas were obtained from a local commercial source.

Seeds were surface sterilized in 0.5% sodium hypochlorite for 10 min, rinsed thoroughly with sterile deionized water and placed in moistened paper towels to germinate. The towels were rolled up, placed upright in 30 ml beakers and the seeds were allowed to germinate for 3 to 5 days at 25 - 27 C in an incubator.

Preparation of Antigens for Serology

Hypocotyls from germinated seeds, singly or in groups of 5 or 10 were tested in double or single radial immunodiffusion tests. If information about the percentage of infected seeds was wanted, 1 - 2 mm thick discs from individual hypocotyls were cut with a razor blade which was rinsed with 95% ethanol and deionized water after cutting each hypocotyl. These hypocotyl discs were tested individually in double or single immunodiffusion tests by embedding them directly into the agar. Groups of 5 or 10 hypocotyls were ground in water or 1.5% SDS (1/1, w/v) with a mortar and pestle as described previously (Purcifull and Batchelor, 1977) and tested against BlCMV and BlCMV-I antisera in double and single radial diffusion. Single or bulked hypocotyls were checked by SSEM for the presence of BlCMV particles.

Double Immunodiffusion Tests

Individual hypocotyls or groups of hypocotyls from germinated cowpea seeds were tested against BlCMV antiserum in double diffusion

tests in agar medium containing 0.8% Noble agar, 0.5% SDS, and 1.0% NaN_3 prepared either in deionized water or in 0.05 M Tris-HCl buffer, pH 7.2. Eight to twelve discs of individual hypocotyls were embedded in the agar medium, 4 to 5 mm away from each antiserum well. Undiluted antiserum for BICMV was routinely used in these tests, but dilutions of 1/2, 1/4, and 1/8 of the antiserum with either normal serum or 0.05 M Tris buffer, pH 7.2 were also tested against hypocotyl discs and extracts of hypocotyl tissue. As controls each hypocotyl was also tested against normal serum, and hypocotyls from noninfected seeds were also included in each test. Extracts from groups of hypocotyls were tested by double immunodiffusion against BICMV antiserum. Hypocotyl extracts were pipetted into the antigen wells distributed in an hexagonal arrangement around the antiserum well. All plates were incubated in a moist chamber at 24 C for 24 to 48 hr. The sensitivity of double immunodiffusion for detection of BICMV in extracts of bulked hypocotyls of germinated 'Knuckle Purple Hull' seeds was tested by mixing 1.0 g of infected hypocotyl tissue with different amounts of noninfected hypocotyls.

Single Radial Immunodiffusion Tests

Discs and extracts of hypocotyls were tested by single radial immunodiffusion in medium containing 0.8% Noble agar, 0.5% SDS, 1.0% NaN_3 and 15% antiserum for BICMV buffered with 0.05 M Tris-HCl, pH 7.2. Hypocotyl discs were embedded directly into the solidified agar medium with the aid of forceps and the plates were incubated in a moist chamber at 24 C for 24 to 72 hr. Precipitin reactions were detected by direct observation of the plates on a darkfield

light box or with a binocular microscope (6 to 15 x) in which the plates were illuminated from the bottom. Extracts of groups of 5 hypocotyls were placed into wells (3 mm in diameter) punched in the agar medium in a row arrangement. The wells were spaced 3 to 4 mm from each other as measured from the edges of the wells and as many as 230 germinated seeds could be tested in a 90 x 15 mm plastic petri dish. Precipitin rings around the wells charged with extracts from infected tissue could be detected anywhere between one to 24 hr.

Serologically Specific Electron Microscopy

Hypocotyl extracts prepared in 0.05 M Tris buffer, pH 7.2, containing 0.15 M NaCl and 0.4 M sucrose were examined for the presence of BlCMV by SSEM as described previously (Derrick and Brlansky, 1976). Copper grids with Parlodion film coated with carbon were treated with BlCMV antiserum diluted to 1/1000 in 0.05 M Tris buffer, pH 7.2. The grids were washed with 0.05 M Tris buffer and floated on drops of hypocotyl extracts for 3 to 4 hr at room temperature. After washing with approximately 2 ml of the extracting buffer and then with approximately 1 ml of deionized water, the grids were positively stained with 1.0% uranyl acetate in 50% ethanol. The grids were washed again with 50% ethanol, dried and examined in the Philips Model 200 electron microscope. The sensitivity of SSEM to detect the presence of BlCMV particles in extracts from a mixture of hypocotyls was determined by diluting infected 'Knuckle Purple Hull' hypocotyl tissue with noninfected tissues up to a dilution of 1/50 (w/w). The different mixtures of infected and noninfected hypocotyl tissues were ground in

extracting buffer (1/1, w/v) with mortars and pestles and small drops from these extracts were used to treat the antiserum-sensitized grids.

Double Immunodiffusion Tests and SSEM for Detection of Other Viruses in Germinated Legume Seeds

Double immunodiffusion tests using discs or extracts of hypocotyls, and SSEM using hypocotyl extracts were used to detect the presence of BCMV and SoyMV in germinated seeds. A seed lot of bean, P. vulgaris 'Black Turtle' containing BCMV-infected seeds was obtained from Dr. Rosario Provvidenti, New York State Agricultural Experiment Station, Geneva. Infected seeds were detected by double immunodiffusion and SSEM using antiserum obtained for a severe strain of BCMV (BCMV-S) isolated from siratro, Macroptilium atropurpureum, in Florida (Lima et al., 1977). Extracts of these germinated bean seeds prepared in 0.05 M potassium phosphate buffer, pH 7.5, were also inoculated in a very sensitive local lesion bean line VC-1822 (Provvidenti and Cobb, 1975). Antiserum for SoyMV was also used to detect SoyMV-infected seeds of soybean, Glycine max.

Serology and Microscopy of Cytoplasmic Inclusions Induced by BlCMV and SoyMV in Hypocotyls of Germinated Seeds

Germinated seeds of cowpea 'Knuckle Purple Hull' infected with BlCMV and germinated seeds of soybean 'Midwest' infected with SoyMV were serologically identified by double immunodiffusion tests using antiserum for BlCMV and SoyMV, respectively, and hypocotyl discs as assay antigens. Hypocotyl extracts from the virus-infected 4-5-day-old seedlings were tested by SDS double immunodiffusion tests against BlCMV and SoyMV inclusion antisera.

Cytoplasmic inclusions were examined in epidermal strips of the hypocotyls of germinated cowpea seeds infected with BlCMV, and of SoyMV-infected hypocotyls of germinated soybean seeds, after staining with a combination of calcomine orange and "luxol" brilliant green (Christie, 1967).

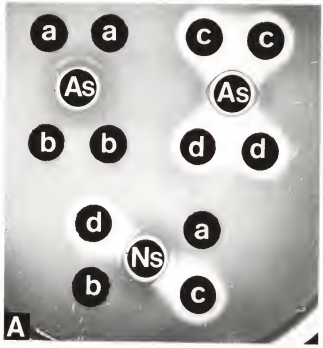
Small pieces of BlCMV-infected and noninfected cowpea hypocotyls were prepared for ultrathin sectioning as described previously in Chapter 1. Similarly, healthy and SoyMV-infected soybean hypocotyls were prepared for ultrathin sectioning. Sections were cut with a diamond knife and stained with potassium permanganate, uranyl acetate, and lead citrate. All specimens were examined with a Philips Model 200 electron microscope.

Results

Preparation of Antigens for Serological Tests

Antigens prepared from hypocotyls of germinated seeds proved to be very satisfactory for indexing seeds in double and single immunodiffusion tests. Neither small discs nor extracts from hypocotyl tissues showed any kind of nonspecific reaction that could interfere with the virus-specific reaction in double or in single immunodiffusion tests. On the other hand, extracts obtained from whole seedlings, including roots, cotyledons and primary leaves showed several types of precipitation patterns when tested against any serum in double diffusion tests (Fig. 19). Such nonspecific precipitations were also observed with extracts obtained from cotyledons, primary leaves or root tissues. These nonspecific reactions were reduced when the agar

Figure 19 - Double immunodiffusion tests with extracts from different portions of BlCMV-infected and healthy, 4-5-day-old cowpea seedlings. A) medium containing 0.8% Noble agar, 1.0% NaN_3, and 0.5% SDS prepared in deionized water. B) medium with the same composition except that it was prepared in 0.05 M Tris-HCl buffer, pH 7.2 instead of water. Center wells were charged with: (As) BlCMV antiserum, and (Ns) normal serum. The peripheral wells were filled with extracts from: (a) hypocotyl from BlCMV-infected cowpea seedlings, (b) hypocotyl from healthy cowpea seedlings, (c) cotyledons and primary leaves of BlCMV-infected seedlings, and (d) cotyledons and primary leaves of healthy cowpea. Note the nonspecific precipitate formed with cotyledons and primary leaves of virus-infected (c) and healthy (d) seedlings and normal serum (Ns) and BlCMV antiserum (As). The virus-specific precipitin lines clearly shown with virus-infected hypocotyls (a) and the antiserum (As) was masked by the nonspecific precipitates when extracts from cotyledons and primary leaves (c) of infected seedlings were used.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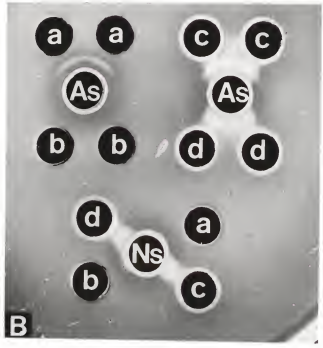
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medium was prepared in 0.05 M Tris buffer, pH 7.2 (Fig. 19-B). Based on these results a diagram showing several procedures for preparation of antigens from legume seeds is suggested (Fig. 20).

Double Immunodiffusion Tests

Blackeye cowpea mosaic virus was detected in hypocotyl extracts from bulked seedlings and in small discs of individual hypocotyls of cowpea 'Knuckle Purple Hull' and 'Early Ramshorn' by double immunodiffusion tests in SDS-gel plates. Virus specific precipitin lines were observed with extracts of mixtures of BlCMV-infected and non-infected cowpea 'Knuckle Purple Hull' hypocotyls up to a dilution of 1/30 (w/w), respectively (Fig. 21-B). This indicated that germinated seeds can be divided into groups of up to 30 seedlings to be tested in double immunodiffusion. Approximately equal amounts of hypocotyl tissue (0.1 - 0.4 g) were cut from each seedling and the extracts obtained from each hypocotyl group were deposited into individual antigen wells. Precipitin reactions were observed with those groups in which at least one seedling was infected with BlCMV. On the other hand, no precipitin reactions were observed with extracts obtained from non-infected hypocotyl tissue, nor with any hypocotyl extract and normal serum (Figs. 19, 21).

When information about the infection percentage was wanted, discs of individual hypocotyls were used in double immunodiffusion. Virus-specific precipitin lines formed between BlCMV-infected hypocotyl discs and antiserum wells, whereas no reactions were observed with non-infected hypocotyls (Fig. 21-C, -D, -F). Good results were observed with undiluted antiserum and with antiserum diluted 1/2 and 1/4 with

Figure 20 - Diagram showing methods for assaying legume seeds by single and double radial immunodiffusion: 1) seeds are placed on moistened paper towels, 2) the towels are rolled up and placed upright in beakers, 3) germinated seeds after four to five days, 4-5) individual seedlings are divided into three parts: roots (r), hypocotyl (hy), and epicotyl (ep) consisting of cotyledons and primary leaves, 6) hypocotyl tissue is ground with mortar and pestle, 7) hypocotyl extracts are tested in double radial immunodiffusion (DRD), 8) hypocotyl extracts are also tested in single radial immunodiffusion (SRD), 9) small discs are cut from each hypocotyl, 10) discs from different hypocotyls are tested by DRD, and 11) discs from different hypocotyls are tested by SRD.

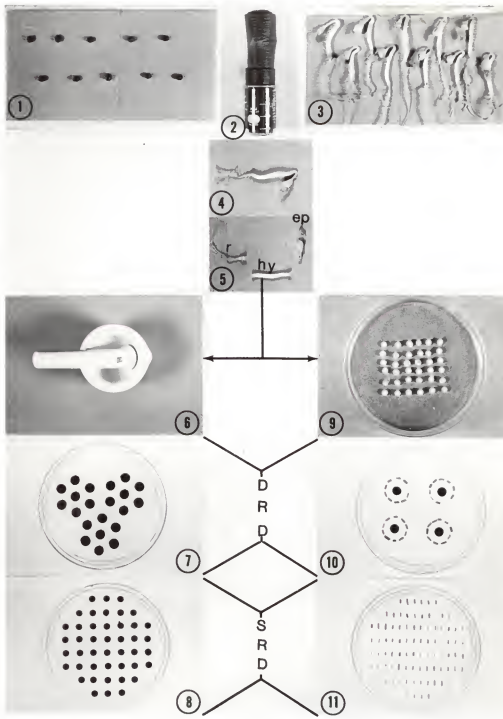
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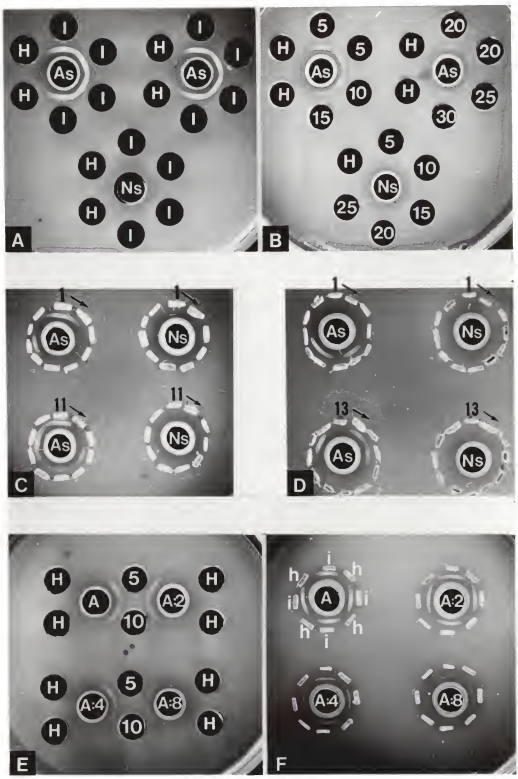
Figure 21 - Double immunodiffusion tests with hypocotyl from healthy and BlCMV-infected, 4-5-day-old cowpea seedlings in medium containing 0.8% Noble agar, 1.0% NaN_3, and 0.5% SDS, prepared in water.

A - B) Serological tests with hypocotyl extracts prepared in water. The center wells were charged with: (As) BlCMV antiserum, and (Ns) normal serum. The peripheral wells were charged with extracts from: (1) BlCMV-infected hypocotyls, (H) healthy hypocotyls, (5) one gram of BlCMV-infected hypocotyl mixed with 4 g of healthy hypocotyls, (10) one gram of BlCMV-infected hypocotyl and 9 g of healthy hypocotyls, (15) one gram of infected hypocotyl and 14 g of healthy hypocotyls, (20) one gram of infected hypocotyl and 19 g of healthy hypocotyls, (25) one gram of infected hypocotyl and 24 g of healthy hypocotyls, and (30) one gram of infected hypocotyl and 29 g of healthy hypocotyls.

C - D) Serological tests with samll discs of different hypocotyls embedded into the agar medium 4 - 5 mm away from the antiserum well. Ten (C) or twelve (D) hypocotyl discs are embedded around the center wells and the hypocotyl discs are numbered from the top in a clockwise direction (arrows). Wells were charged with: (As) BlCMV antiserum, and (Ns) normal serum. Note virus-antiserum specific reactions with hypocotyls: 1, 2, 8, 11, 14, 16, 18, 19 (C), and 6, 8, 9, 14, 16, 20, and 23 (D).

E - F) Serological tests with hypocotyl extracts (E) and hypocotyl discs (F) from healthy and BlCMV-infected cowpea seedlings. The center wells were charged with: (A) undiluted BlCMV antiserum, (A:2) BlCMV antiserum diluted 1/2 with normal serum, (A:4) BlCMV antiserum diluted 1/4 with normal serum, and (A:8) BlCMV antiserum diluted 1/8 with normal serum. The peripheral wells were charged with extracts from: (5) one gram of BlCMV-infected hypocotyls, and 4 g of healthy hypocotyls, (10) one gram of BlCMV-infected hypocotyls and 9 g of healthy hypocotyls, and (H) healthy hypocotyls. Hypocotyl discs from healthy (h) and BlCMV-infected (i) seedlings were alternated around the antiserum wells in F.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A:8
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h
i

normal serum (Fig. 21-F). The percentages of BlCMV-infected seeds in four different lots of cowpea 'Knuckle Purple Hull' and one 'Early Ramshorn' seed lot were estimated by this simplified double immunodiffusion test and by the growing-on test (Table II). Over a hundred seedlings tested by this serological technique were randomly checked by SSEM for the presence of BlCMV particles and in all cases the results corresponded.

Single Radial Immunodiffusion Tests

The presence of BlCMV in cowpea hypocotyl extracts was detected by single radial immunodiffusion tests in agar medium impregnated with BlCMV antiserum. Precipitin rings were observed around the wells charged with extracts from groups of 5 individual hypocotyls in which at least one was infected with BlCMV, but not around those wells charged with extracts of noninfected hypocotyls (Fig. 22). The virus-specific reactions started to appear at approximately one hour after the hypocotyl extracts had been added into the wells and became very clear and evident 3 to 23 hr later. The reactions were still very distinct 24 hr after the hypocotyl extracts had been added into the wells.

When small discs of individual hypocotyls were directly embedded into the agar medium containing BlCMV antiserum, the virus-specific reactions took longer to appear. These specific reactions were recognized as opalescent precipitates around infected hypocotyl discs that could be better detected under a binocular microscope. The opalescent precipitates which usually were located at the ends of the hypocotyl discs, started to appear in 24 hr, but were more evident

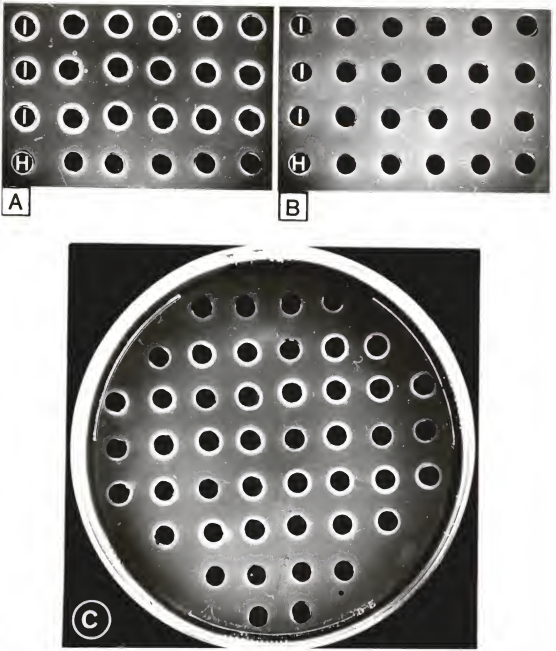
Table II - Comparison of immunodiffusion tests with hypocotyl discs and growing-on tests for detection of virus-infected seeds.

Seed Source	Virus Tested	Seed Assay Methods			
		Serology		Growing-on Test	
		Proportion of Infected Seeds	Percentage of Infected Seeds	Proportion of Infected Seeds	Percentage of Infected Seeds
Cowpea (V. unguiculata)					
Knuckle Purple Hull					
Lot A	BlCMV	72/323*	22.3	33/160	20.6
Lot B	BlCMV	12/160	7.5	13/173	7.5
Lot C	BlCMV	5/115	4.3	8/145	5.5
Lot D	BlCMV	17/160	10.6	13/141	9.2
Early Ramshorn	BlCMV	5/100	5.0	34/545**	6.3**
Soybean (G. max)					
Jupiter	SoyMV	4/176	2.8	2/89	2.2
Bean (P. vulgaris)					
Black Turtle	BCMV	3/79	3.8	Not tested	Not tested

(*) - Number of virus-infected seeds over number of seeds tested;

(**)- Pio-Ribeiro, G. (unpublished).

Figure 22 - Single radial immunodiffusion tests with hypocotyl extracts from healthy (H) and BICMV-infected (I), 4-5-day-old cowpea seedlings in media containing 0.8% Noble agar, 1.0% NaN_3, 0.5% SDS, and 15% BICMV antiserum (A, C) and 15% normal serum (B). The top row and the two bottom rows of wells in C were charged with extracts from healthy hypocotyls and the others were randomly filled with extracts from groups of 5 hypocotyls containing 1 or 2 BICMV-infected hypocotyl per group.


A
B
C

at 48 to 72 hr after the test had been set up. Usually after 72 hr, a nonspecific precipitate also started to appear throughout the agar medium.

Serologically Specific Electron Microscopy

The SSEM technique developed by Derrick and Brlansky (1976) was adapted with great success to identify seed lots of cowpea infected with BlCMV (Fig. 23). Virus particles were still observed in extracts from hypocotyl tissue which was mixed 1/50 (w/w) with noninfected hypocotyls (Fig. 24-B). This indicates that to test several seed lots by SSEM for the presence of BlCMV, germinated seeds can be divided into groups of up to 50 equal pieces of individual hypocotyls. Each group of hypocotyl-pieces is ground in the extracting buffer and a drop of the extract is then examined by SSEM using antiserum sensitized grids. Virus particles will be observed in extracts obtained from those hypocotyl groups in which at least one hypocotyl is infected with BlCMV.

To illustrate the sensitivity of SSEM for detection of virus particles in hypocotyl extracts, the same BlCMV-infected hypocotyl was used for four different electron microscopic preparations: a) SSEM; b) normal dip preparation on a carbon coated Formvar film supported in copper grids and negatively stained with 2% PTA; c) preparation similar to SSEM using normal serum instead of BlCMV-antiserum; and d) preparation similar to SSEM using grids with Parlodion film coated with carbon but treated with 0.05 M Tris buffer instead of antiserum (Fig. 23). The results showed a great increase of virus concentration on the grids sensitized with BlCMV antiserum when compared with the

Figure 23 - Electron micrographs of BlCMV in hypocotyl extracts from the same cowpea seedlings using different preparations. (A) serologically specific electron microscopy (SSEM) with BlCMV antiserum diluted 1/1000 in 0.05 M Tris-HCl buffer, pH 7.2, and positively stained with uranyl acetate, (B) dip preparation with 2% phosphotungstic acid pH 6.5, containing 0.1% BSA, (C) preparation similar to SSEM using normal serum instead of BlCMV-antiserum, and (D) preparation similar to SSEM using Tris buffer instead of antiserum. The micrograph A represents a typical view of the entire grid whereas micrographs B, C, and D are selected areas of the grids showing virus particles (arrows).

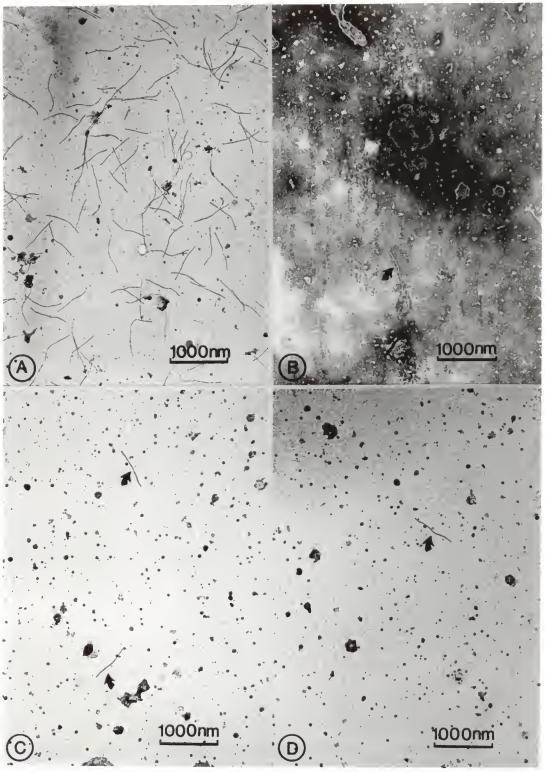

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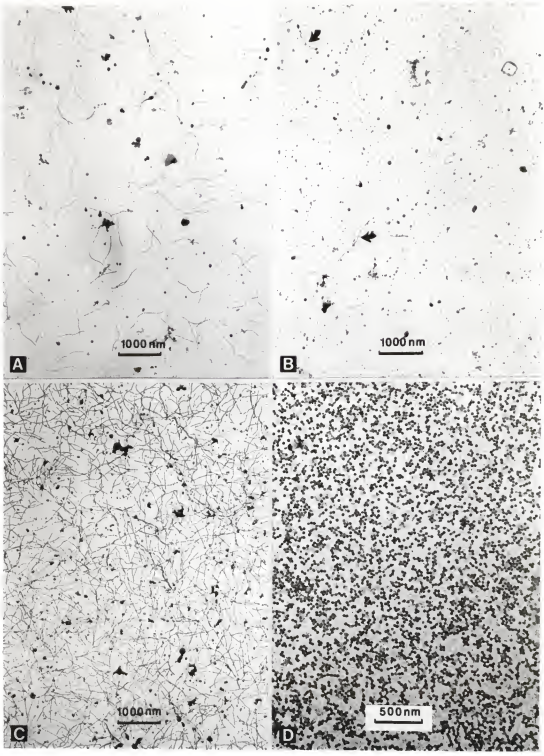
Figure 24 - Electron micrographs of serologically specific electron microscopy with BlCMV, BCMV-S, and CPMV.

A) BlCMV antiserum grid and extracts from a BlCMV-infected cowpea hypocotyl;

B) BlCMV antiserum grid and extracts from one gram of BlCMV-infected hypocotyl and 49 g of healthy cowpea hypocotyls. Arrows point to virus particles;

C) BCMV-S antiserum grid and leaf extract from BCMV-S infected bean;

D) CPMV antiserum grid and leaf extract from CPMV-infected cowpea.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other grid preparations (Fig. 23). As the antiserum sensitized grids were washed several times during their preparation, a great reduction in the amount of plant debris on the grids was observed. On the other hand, when grids not previously treated with the antiserum were washed, the virus particles were also removed (Fig. 23-C, -D).

The SSEM seemed to be also adequate to assay seeds for polyhedral viruses since it was successfully used to observe CPMV particles in cowpea leaf extracts using grids treated with antiserum specific for CPMV (Fig. 24-D).

Double Immunodiffusion Tests and SSEM for Detection of Other Viruses in Germinated Legume Seeds

The double immunodiffusion tests and SSEM used to detect BlCMV in germinated cowpea seeds were also useful for detecting BCMV in infected germinated bean seeds and SoyMV in hypocotyls of germinated soybean seeds infected with SoyMV. Both legume viruses were detected by double immunodiffusion tests using hypocotyl extracts from groups of 5 seedlings and small discs of individual hypocotyls as assay antigens (Fig. 25). The results obtained in the simplified double immunodiffusion tests with hypocotyl discs were confirmed by SSEM, which also proved to be a very good technique to assay bean and soybean seed lots for the presence of BCMV (Fig. 26-A) and SoyMV (Fig. 26-B), respectively.

The results obtained from the inoculation of the hypersensitive bean line VC-1822 with extracts from germinated bean seeds were also in agreement with those obtained by the immunochemical tests for BCMV. The percentages of BCMV-infected bean 'Black Turtle' seed lot and of

Figure 25 - Double immunodiffusion tests with hypocotyls of 4-5-day-old bean and soybean seedlings using antiserum for BCMV-S and for SoyMV.

A - B) Serological tests with hypocotyl discs (A) and hypocotyl extracts (B) from healthy and BCMV-BV-1-infected bean seedlings. The center wells were charged with: (As) BCMV-S antiserum, and (Ns) normal serum. The peripheral wells were filled with extracts from: (1) BCMV-BV-1-infected hypocotyls, and (H) healthy bean hypocotyls. The hypocotyl discs were embedded directly into the agar medium and numbered from the top in a clockwise direction (arrows). Note a virus-antiserum specific reaction with hypocotyl "1" in A.

C - D) Serological tests with hypocotyl discs (C) and hypocotyl extracts (D) from healthy and SoyMV-infected soybean seedlings. The center wells were charged with: (As) SoyMV antiserum, and (Ns) normal serum. The peripheral wells were filled with extracts from: (1) SoyMV-infected hypocotyls, (2) SoyMV-infected Nicotiana benthamiana leaves, (3) healthy N. benthamiana leaves, and (4) healthy soybean hypocotyls. Note a positive reaction with hypocotyl "14" in C.

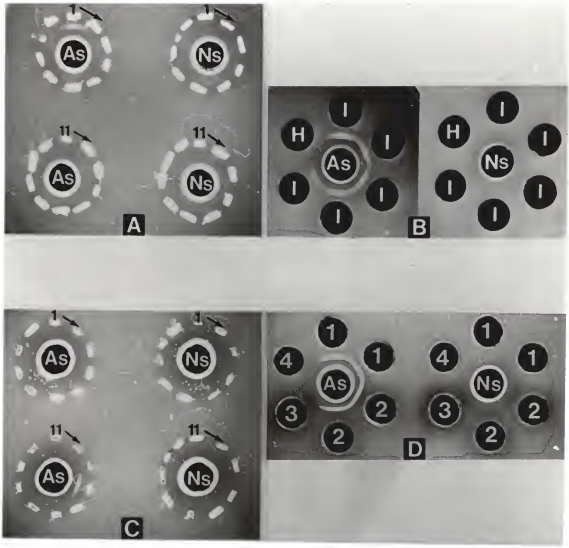
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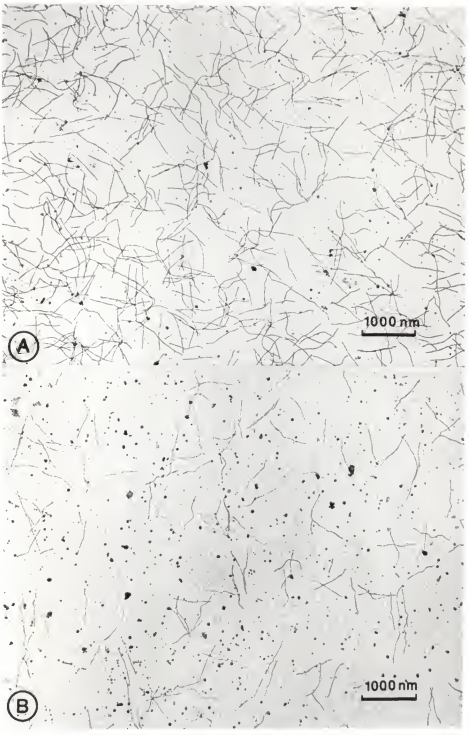
Figure 26 - Electron micrographs of serologically specific electron microscopy with extracts from BCMV- and SoyMV-infected hypocotyls.

A) BCMV-S antiserum-sensitized grid and extract from bean hypocotyl infected with BCMV-BV-1;

B) SoyMV antiserum-sensitized grid and extract from SoyMV-infected soybean hypocotyl.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1000 nm
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SoyMV-infected soybean 'Jupiter' seed lot were also estimated by the simplified double immunodiffusion test using hypocotyl discs (Table 11).

Serology and Microscopy of Cytoplasmic Inclusions Induced by BlCMV and SoyMV in Hypocotyls of Germinated Seeds

Cytoplasmic inclusions induced by BlCMV in cowpea and by SoyMV in soybean were detected by serology, light microscopy and electron microscopy in hypocotyls of 4-5-day-old seedlings. In double immunodiffusion tests, specific precipitin lines were observed with the inclusion antisera and extracts of virus-infected hypocotyls but not with extracts from healthy hypocotyls (Fig. 27).

Light microscopic observations of epidermal strips of hypocotyls from BlCMV-infected cowpea and SoyMV-infected soybean seedlings readily revealed the presence of cytoplasmic inclusions (Figs. 28, 29). Groups of inclusions induced by BlCMV and by SoyMV were abundant in cells of virus-infected hypocotyls (Figs. 28, 29), but were not observed in cells of noninfected hypocotyls. Pinwheels with scrolls were observed in ultrathin sections of hypocotyl tissues infected either with BlCMV or SoyMV (Figs. 30, 31). A large number of longitudinal sections of pinwheel inclusions in hypocotyl tissues showed that they were abutted to the cell wall close to the plasmodesmata (Fig. 31-C, -D). Similarly, tubes were observed in molybdate-treated extracts from hypocotyls infected with either virus.

Figure 27 - Double immunodiffusion tests with hypocotyl extracts from 4-5-day-old seedlings of cowpea (A) and soybean (B) using antisera for BlCMV, SoyMV, and their cytoplasmic inclusions.

A) Serological test for detecting BlCMV cytoplasmic inclusions (BlCMV-I) in hypocotyls of 4-5-day-old cowpea seedlings grown from BlCMV-infected seeds. The center wells were charged with: (Is) BlCMV-I antiserum, (Vs) BlCMV antiserum, and (Ns) normal serum. The peripheral wells were charged with SDS-treated extracts from: (I) BlCMV-infected hypocotyls, and (H) healthy hypocotyls.

B) Serological test for detection of SoyMV cytoplasmic inclusions (SoyMV-I) in hypocotyls of 4-5-day-old soybean seedlings grown from SoyMV-infected seeds. The center wells were charged with: (Is) SoyMV-I antiserum, (Vs) SoyMV antiserum, and (Ns) normal serum. The peripheral wells were charged with SDS-treated extracts from: (I) SoyMV-infected hypocotyls, and (H) healthy soybean hypocotyls.


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Figure 28 - Photomicrographs showing different views (A, B, C, D) of cytoplasmic inclusions (arrows) induced by BICMV in epidermal strips of cowpea hypocotyl tissue stained with a combination of calcomine orange and luxol brilliant green. (CI) cytoplasmic inclusions, (CW) plant cell wall, and (Nu) nucleus.

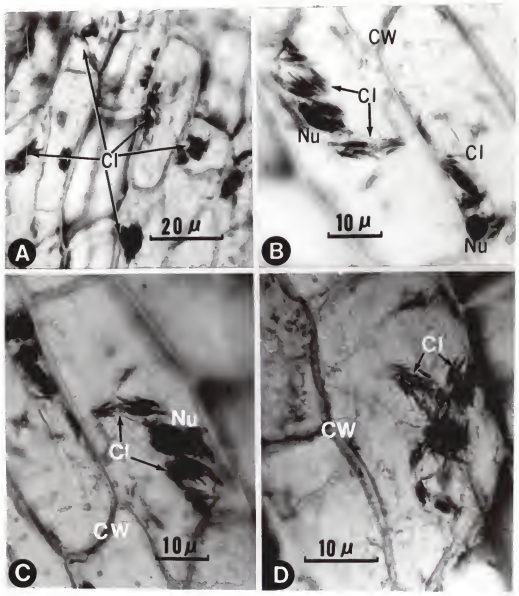
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Figure 29 - Photomicrographs showing different views (A, B, C, D) of epidermal cells of hypocotyls from 4-5-day-old soybean seedlings containing cytoplasmic inclusions (arrows) induced by SoyMV. The hypocotyl epidermal strips were stained with a combination of calcomine orange and luxol brilliant green. (CI) cytoplasmic inclusions, (CW) plant cell wall, and (Nu) nucleus.

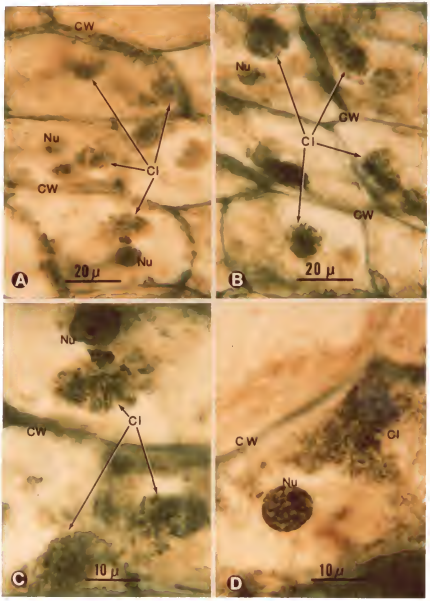
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Figure 30 - Electron micrographs of ultrathin sections of cells from hypocotyls of 4-5-day-old cowpea seedlings infected with BlCMV. Note cross-sections (A, B, C) and longitudinal sections (D) of pinwheel inclusions induced by BlCMV. (CW) plant cell wall, (IS) intercellular space, (m) mitochondrion, and (pw) pinwheel inclusions.

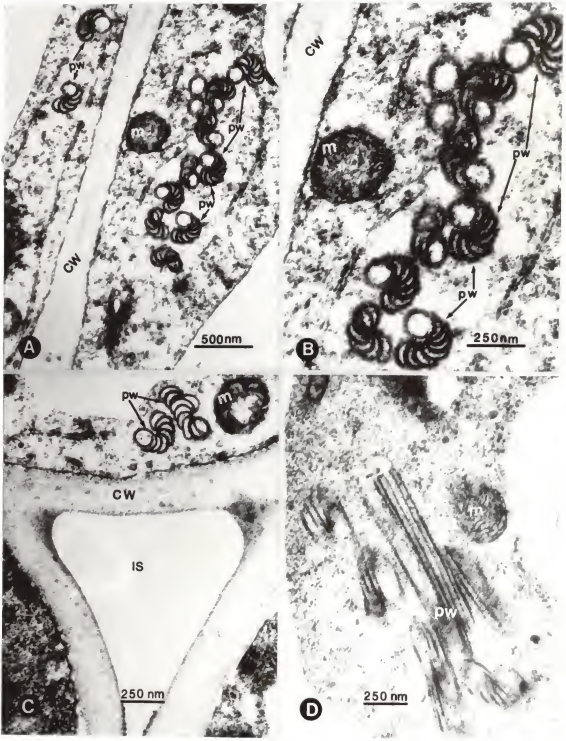
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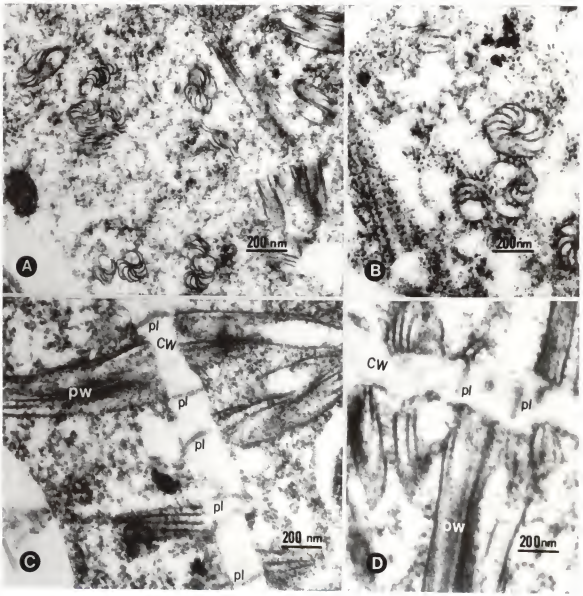
Figure 31 - Electron micrographs of ultrathin sections of hypocotyl cells of 4-5-day-old soybean seedlings grown from SoyMV-infected seeds. Note cross-sections (A, B) of the pinwheels and longitudinal views of the inclusions abutted to the plant cell wall at the plasmodesmata (C, D). (CW) plant cell wall, (pl) plasmodesma, and (pw) pinwheel inclusions.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Discussion

Seed-transmission of plant viruses is of agricultural importance because efficient virus transmission in space and time can be provided through seeds of infected host plants. The international exchange of several legume seeds is probably partly responsible for the worldwide distribution of important virus diseases of legumes. The occurrence of SoyMV in soybean and BCMV in bean in most countries where these crops have been tested for viruses (Bos, 1971, 1972) are good examples to illustrate this point. According to Phatak (1974), several legumes are propagated in association with their seed-borne viruses on a wide scale in certain subtropical and tropical regions that lack organized seed certification and breeding programs. Brunt and Kenten (1973) explained the prevalence of cowpea mild mottle virus in cowpea in Ghana by the high rate of seed transmission in cowpeas and by the traditional practice of sowing seeds from crops previously grown in the same region.

The control of virus diseases of legumes through the production of virus-free seeds or seed lots with very low percentage of infected seeds has been advocated frequently (Fajardo, 1930; Thomas and Graham, 1951; Zettler and Evans, 1972; Baker, 1972; Phatak, 1974; Sinclair and Shurtleff, 1975; and Williams, 1975). The increase in percentage of infected seeds produced in crops with low initial infection and the efficient secondary spread of certain viruses into the growing crop from a small amount of primary inoculum randomly located throughout the planting, emphasize the importance of maintaining a low tolerance level in seed certification programs.

Several techniques have been developed for diagnosing seed-borne viruses in routine seed health testing laboratories. Phatak (1974) grouped those techniques into dry examination (visual inspection of dry seeds), biological tests (growing-on and infectivity tests), biochemical tests (colorimetric, histochemical, and serological tests), and biophysical tests (electron microscopy). According to Phatak (1974) the highly specific serological tests are the best among the tests available to assay seed lots for the presence of virus-infected seeds. Several serological tests have been used for detecting virus-infected seeds (Scott, 1961; Hamilton, 1965; Phatak, 1974; Slack and Shepherd, 1975; Lundsgaard, 1976; and Lister, 1977). However, the present investigation contains the first successful results with double immunodiffusion tests for detecting potyviruses in germinated seeds.

The use of hypocotyl tissue of germinated legume seeds eliminated the problem of nonspecific reaction commonly observed with extracts from seeds per se or germinated seeds, including roots, cotyledons, and primary leaves in immunodiffusion tests (Fig. 19). Cockbain et al. (1976) reported that when embryos of Vicia faba L. minor seeds were tested by double immunodiffusion, the agar became clouded, obscuring the virus-specific precipitation lines. The nonspecific precipitates that interfered with the virus-specific reactions (Fig. 19) may be related to the high concentration of haemagglutinins (lectins) reported to be present in legume seeds (Toms and Turner, 1965; Moreira and Perrone, 1977; and Fountain and Yang, 1977). It has been demonstrated that lectins bind specifically to mono and polysaccharides as

well as to globulins of normal serum and to virus coat protein. Marshall and Norins (1965) showed that extracts of P. vulgaris seeds containing lectins precipitated α and β globulins of normal rabbit serum. Gumpf and Shannon (1977) demonstrated that a barley lectin interacted with purified BSMV in vitro and formed insoluble aggregates that greatly reduced the virus infectivity. Phatak (1974) assumed that the high content of lectin in soybean seeds was responsible for the unsatisfactory results of the passive haemagglutination test with SoyMV in seed extracts. Thus, the absence of nonspecific reactions with extracts of hypocotyl tissue in contrast to the nonspecific precipitates observed with extracts of root or cotyledons and primary leaves (Fig. 19) of 4-5-day-old legume seedlings may be an indication that a very low content of lectin is present in the hypocotyl.

In support of the use of germinated seed for serology, instead of seed per se, is the fact that if any seed certification program or seed producers will assay for the presence of virus-infected seeds, they will also test some other seed properties such as percentage of seed germination. Consequently, the germinated seeds could be used in a concomitant serological indexing program.

The sensitivity of the double immunodiffusion test indicates that germinated seeds can be divided into groups of 5 to 30 seedlings and precipitin reactions will be observed with those groups containing at least one infected seedling (Fig. 21). Immunochemical tests with higher sensitivity have been also used for indexing virus-infected seeds. The extremely sensitive enzyme-linked immunosorbent assay (ELISA) (Voller et al., 1976; and Clark and Adams, 1977) was used to

detect TRSV and SoyMV in individual soybean seeds (Lister, 1977). The high sensitivity of SSEM for detection of particles of BlCMV, BCMV, and SoyMV in infected hypocotyl extracts as well as of other seed-borne viruses in seed extracts (Brlansky and Derrick, 1976) make it suitable for identification of virus-infected seeds when an electron microscope is available. The ELISA and SSEM techniques, however, are more complex than the double immunodiffusion tests and/or require the use of sophisticated equipment such as an electron microscope.

A close correlation was observed between the percentages of virus-infected seeds estimated by the simplified double immunodiffusion technique with discs of individual hypocotyls and the growing-on test in different legume seed lots (Table II). The advantages of the hypocotyl disc-double immunodiffusion technique over the growing-on test are that: a) it is faster and more specific, b) it does not require greenhouse space, and c) it does not depend on environmental conditions for symptom development. The growing-on test is largely used, sometimes in combination with serology, for indexing seed lots for the presence of virus-infected seeds. The growing-on test, however, requires suitable temperature and light under insect-proof conditions for a period varying from 2 to 6 weeks. Kaiser and Mossahebi (1974) reported that at times, virus symptoms did not appear until the second or third trifoliolate leaf showed up on bean plants grown from seeds infected with BCMV. According to Sharma and Varma (1975), the symptoms of cowpea banding mosaic virus appeared only on the first trifoliolate or the subsequent leaves of cowpea under low temperature conditions. Tosic and Pesic (1975) observed that the greatest number of alfalfa seedlings

infected with alfalfa mosaic virus could be detected only 14 days after germination and symptom expression was not reliable enough for the estimation of percentage of virus-infected seeds. Phatak et al. (1976) used the indicator-inoculation test in association with the growing-on test to estimate the percentage of cowpea seeds infected with cowpea ringspot virus because infected seedlings were either symptomless or the symptoms were very mild. In assessing the proportion of V. faba minor seeds infected with broad bean stain virus, Cockbain et al. (1976) found that under high temperature the symptoms were often mild and evanescent, and virus-infected seedlings remained without obvious symptoms for more than 6 weeks. Thus, the quantitative estimation of virus-infected seeds of leguminous crops by the growing-on test may often be difficult due to symptomless infections. On the other hand, the reliability and simplicity of the hypocotyl-disc-double immunodiffusion test make it highly suitable for commercial certification programs. Observations during the course of this research indicated that a trained person can set up approximately 50-60 hypocotyl discs per hour in an agar plate to be tested by this technique. This simplified double immunodiffusion technique does not require tissue grinding, antigen wells, and chemical treatment of the antigen prior to incorporation into the agar matrix.

The single radial immunodiffusion test also proved to be satisfactory for detection of BICMV in cowpea hypocotyls. These results provide another option for a routine seed health testing program. Single radial immunodiffusion in multiple-antisera media could be used for indexing legume seeds for more than one virus. As shown in the

first part of this research, an agar medium impregnated with a mixture of antisera was used for serodiagnosis of two morphologically distinct viruses in cowpea (Figs. 13, 14).

The presence of cytoplasmic inclusions in tissues of germinated seeds is also useful for detection of virus-infected seeds by light microscopy and serology. Cytoplasmic inclusions induced by BlCMV in cowpea and by SoyMV in soybean were detected by serology, light microscopy and electron microscopy in hypocotyls of germinated seeds. Although the cytoplasmic inclusions induced by potyviruses were found to be widespread in systemically infected plants (Sheffield, 1941; Hampton et al., 1973; and Tu, 1973), no previous report has indicated their presence in either infected seed per se or in germinated virus-infected seeds (Sheffield, 1941; Camargo et al., 1968; Edwardson, 1974; Andrews and Shalla, 1974; Tu, 1975; Martelli and Russo, 1977; and Christie and Edwardson, 1977).

In summary, the following techniques have been demonstrated to be useful for detecting potyviruses and/or their cytoplasmic inclusions in hypocotyl tissues of 4-5-day-old seedlings grown from virus-infected legume seeds: a) conventional double immunodiffusion, b) hypocotyl-disc-double immunodiffusion, c) conventional single radial immunodiffusion, d) hypocotyl-disc-single radial immunodiffusion, e) SSEM of virus particles; and g) light microscopy of cytoplasmic inclusions. The hypocotyl-disc-double immunodiffusion technique is the simplest method to use when it is necessary to determine the percentage of virus-infected seeds.

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BIOGRAPHICAL SKETCH

José Albersio de Araujo Lima was born July 12, 1940, in Santana do Cariri, Ceará, Brazil. He graduated from high school in December, 1959, at the Colégio Estadual do Ceará in Fortaleza, Ceará, Brazil. He joined the Army in December, 1958, and was honorably dismissed as an Army Reserve Officer in December, 1960. He attended the Universidade Federal do Ceará, receiving the Bachelor of Science degree in Agronomy, along with a diploma of Agronomic Engineering, in December, 1966. In March, 1967, he was hired as an Auxiliary Professor of Plant Pathology by the Universidade Federal do Ceará, and in 1973, he was promoted to Assistant Professor, a position that he has held up to the present time. He started a graduate program in Plant Pathology at the University of Arizona, Tucson, in September, 1970, and received his Master of Science degree in 1972. In September, 1974, he entered the University of Florida, receiving the Doctor of Philosophy degree in March, 1978. He is the author or co-author of approximately ten publications of researches in Plant Pathology.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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